

Bacteria Isolated From Sunff (*Nicotiana rustica*) Before and after Fermentation

By

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بسم الله الرحمن الرحيم

قال الله تعالى(وفوق كل ذي علم عليم)

صدق الله العظيم

يوسف:(76)

(وما أوتيتم من العلم لا قليلا)

صدق الله العظيم

الأسراء: (85)

DEDICATION

TO

My mother, father, brothers and sisters

I dedicate this work

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ABSTRACT

The present study was planned to isolate and identify the types of bacteria present in toombak .Sixty six samples of moist and dry snuff were collected from different toombak shops in Khartoum, Omdurman, and Khartoum north. The samples consisted of fifty moist samples and sixteen dry samples.

The samples were subjected to bacteriological examination to investigate aerobic and anaerobic bacteria.

No bacterial growth was obtained when the samples were cultured anaerobically, while 195 bacterial isolates were obtained aerobically from 66 toombak samples. 193 isolates were Gram positive bacteria predominantly thirty isolates (15,5%) were *Bacillus mycoides* ,39 isolates (20,2%) were *B. amyloliquefaciens*,37 isolates (19,2%) were *B. subtilis*,12 isolates (6,2%) were *B. cereus*,17 isolats (8,8%) were *B. thuringiensis*,16 isolates (8,2%) were *B. sphaericus*, 14 isolates (7,3%) were *B. megaterium*, 9 isolates (4,7%) *B. coagulans*, 9 isolates (4, 7%) were *B. licheniformis*, nine isolates (4, 7%) were *B. brevis*, two isolates (1, 0%) were *B. alvei* and one isolate (5%) was *B. pantothenicus*.

While the total of Gram-negative bacteria was, only two isolates belonged to *Providencia.rettregi* species. In this study, we measured the level of pH and the redox potential of moist, dry and natron and redox potential after addition of cystein hydrochloride to moist snuff solution was also measured which became more reduced.

ملخص الأطروحة

هذه الدراسة أعدت لتمييز الأنواع البكتيرية الموجودة في التبناك . 66 عينة من التبناك الرطب والجاف جمعت من دكاكين مختلفة في الخرطوم والخرطوم بحري وأم درمان . أشتملت على 50 عينة رطبة و16 عينة جافة .

أعدت هذه العينات للفحوصات البكتيرية الهوائية واللاهوائية حيث لا نمو في العينات التي تمت زراعتها لا هوائياً بينما كان عدد العزولات البكتيرية الهوائية 195 حصلت من 66 عينة تبناك منها 193 عزل بكتيريا موجبة لصبغة الجرام عبارة عن *Bacillus species* متمثلة في 30 عزل (51.5%) *B. mycoida* و39 عزل (20.2%) *amyloliquefaciens* و37 عزل (19.2%) *B. subtilis* و12 عزل (6.2%) *B. cereus* و17 عزل (8.8%) *B. thuringiensis* و16 عزل (8.2%) *B. sphaericus* و14 عزل (7.3%) *B. megaterium* و9 عزل (4.7%) *B. coagulans* و9 عزل (4.7%) *B. licheniformis* و2 عزل (1.0%) *B. alvei* و1 عزل (0.5%) *B. pantothenicus* و9 عزل (4.7%) *B. brevis*.

وأما مجموع البكتيريا السالبة لصبغة الجرام 2 فقط متمثلة في *Providencia rettgi* عزلت من العينات الجافة .

وفي هذه الدراسة قمنا بقياس مستوى درجة الحموضة pH ومعامل الأكسدة والإختزال Eh في عينات التبناك الرطب والجاف والعطرون وقياس معامل الأكسدة والإختزال Eh بعد إضافة cystein hydrochlorid إلى عينة التبناك الرطب مما جعل المحلول أكثر إختزالاً.

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Introduction

The oral use of smokeless tobacco either in the form of snuff as used in North America and Western Europe or mixed with lime and areca nut in the form of a betel quid as used in Asia has been unequivocally associated with human cancers, mainly of the oral cavity. However, little has been published about smokeless tobacco as it is used in Sudan and other African countries and any possible association with neoplastic diseases.

In the Sudan the prevalent oral use of smokeless tobacco is in the form of snuff (saffa, toombak in local language), prepared from sun-dried tobacco leaves. The main tobacco species is *Nicotiana rustica*, the leaves of which are usually mixed with aqueous solution of natron (sodium bicarbonate). An alternative base to slaked lime used in other parts of the world by tobacco chewers until saturated, the product left in a closed container for about 24 hr before use.

A study at the River Nile state at the North of Sudan revealed that the prevalence of toombak dipping and cigarette smoking among men and women is in the range of 25-47% and 13-25% respectively.

The use of snuff increases risk of cancer, also it is associated with oral mucosal lesions, including keratosis, oral precancer, leukoplakia, and other oral diseases such as gingival recession, dental caries.

Our studies have been shown that toombak and saliva of toombak dippers contain unusually high levels of the carcinogenic tobacco specific-N-nitrosamines (TSNA) higher than those reported from snuff products that used in Western Europe and North America. We choosed this interesting

topic due to prevalence of toombak use in Sudan and its impact on health, social and economical situations.

The aim of this overview was to cover the general profiles of these products and provide any available information on populations using them, to know any published work on the risk of oral and other cancers and other oral health risks, carcinogenicity, clinical, histological and biomarker of toombak dipper's lesions and toombak related carcinomas including expression and mutations of p53 gene, presence of HPV infection in toombak user's leads to expression of keratins 13, 14, and 19 which are differentiation markers in oral cancer and precancerous lesions.

Objectives

1. To study the microbial load of toombak before and after addition of natron.
2. To highlight the ability of the isolated bacteria in releasing virulence factors leading to oral and abdominal disturbances and injuries.
3. To measure the pH and redox potential of dry toombak, moist toombak and natron, in relation to the growth of anaerobic bacteria in toombak mucosa.
4. To study the involvement of the isolated bacteria in cancer formation.

CHAPTER ONE

Literature review

1-1: Definition of toombak:

In the Sudan, snuff locally known as toombak was introduced approximately 400 years ago. It is always processed into loose moist form, and its use widespread in the country. Tobacco used for manufacturing of toombak is of species *Nicotiana rustica*. The fermented ground powder is mixed with an aqueous solution of sodium bicarbonate (Idris *et al.*, 1998).

Introduction of this tobacco plant to the Sudan was attributed to a koranic (Islamic) teacher who came to the Sudan, either from Egypt, Timbuktu of Mali or Morocco. It has also been suggested that toombak was introduced to the Sudan from Turkey or Arabia means sniffing of the product in the local language indicating nasal usage when it was first introduced. The commercial names for toombak include, El-sanf (of high quality) wad Amari (according to the person who was believed to have introduced it) and Sultan El- kaif) the power to improve one's state of mind.

Tobacco is primarily consumed in the Sudan in two forms oral snuff and cigarettes. Oral snuff is consumed as twice as cigarettes and named toombak in the local language, is home-made from finely ground leaves of *Nicotiana rustisa*. Tobacco species with an especially higher content of levels of alkaloid (nicotine, anabasine, nornicotine) than *Nicotiana tabcum* used for cigarettes (Idris *et al.*, 1995), which a prime factor for popularity of tobacco.

Smokeless tobacco product (toombak) has been used in the Sudan for centuries and is widespread, especially in the northern, eastern and central parts (Idris *et al.*, 1994). The use of toombak is particularly common among the Gaalen and Shiagia tribes who reside these regions (EL-Besheir *et al.*, 1989). So far only one study has estimated the prevalence of use of toombak in the River Nile province in the north of Sudan (Idris *et al.*, 1994) by 40% among adult male dip toombak including 9% who are also cigarette smokers among men aged 40 years or older. From many surveys performed randomly in the river Nile stat to estimate the prevalence of tobacco use they found that among children and adolescents (4-7) was quite low (2%-1-2%) but there was an abrupt increase up to (25%) in late adolescents. Among the adult population aged 18 year and older the prevalence of toombak use (34%) and cigarette smoking (12%). Among males were significantly higher than among females (2.5% and 0.9% respectively). The prevalence of toombak use among the male population aged 18 years and older was significantly higher in the rural than in the urban areas (35%to 24%), while cigarette smoking had a higher prevalence in urban areas (18%to 12%). The highest rates of toombak use were found in rural areas among the male population ages 30 years and older (Idris *et al.*, 1998).

1.2. Classification of tobacco plant:-

The genus *Nicotiana* is classified among the family *Solanaceae* which comprises about 100 species. The most famous species is the largely cultivated *Varinia. tobacco*, *Nicotiana. tabacum*, *Turkish tobacco* and *Nicotina rustica* (Broun and Massey., 1929).

Tobacco is believed to be native of tropical America and was cultivated and used by native inhabitants before the discovery of America. It is the one of the few major contributions to civilization which the new world can claim. The first who used tobacco were the Indian of north and South America and spread to other countries France 1556, England 1565 and from these countries to the different parts of the world (Hussain 1984).

The ancestry of *Nicotiana tobacum* is not known. Nicotine is the main alkaloid of the genus and is prepared commercially from waste material of the tobacco industry.

1.3. Family Solanaceae:-

Herbaceous or woody plant. Leaves are without stipules, alternate, simple flowers, hermaphrodites or very rarely unisexual. Usually actinomorphic, calyx 4-6 lobed persistent corolla gamopetalous usually five lobed flared, contorted or valvate stamens inserted on the corolla lobes rarely two anther loculi parallel, ovary usually two locular. The loculi sometimes divided by a false septum style terminal. Ovule very numerous exiles, Fruity capsule or berry (Andreus, 1951).

1.4. *Nicotiana. rustica*:-

It is semi desert plant, grows in different areas in the Sudan but mainly in Darfour at the western region (Hiday – talla, 1983). Herb is up to four feet high. Leaves petiolate ovate obtuse at the apex, some times subcordate at the base, up to one foot high long glandular pubescent. Flowers greenish yellow, in terminal subpaniculate. Racemes with or

without bract .Capsule subglobose slight longer than the calyx (Broun and Massey, 1929).

1.5. Chemical Composition of Tobacco:-

The active ingredient in tobacco is alkaloids of naturally occurring compound containing nitrogen and having the properties of an aminebase, they have dramatic effects on the human system (Hommond, 1962). It was first isolate from genus nicotiana in 1828 (Pavia *et al.*, 1976), nicotine is a colorless oily liquid alkaloid, and it considers of the most toxic drugs known to human, a dose of 60 mg is lethal in a few minutes (Pavaia *et al.*, 1976). Hussain, (1984) reported that nicotine constitutes 0, 9 to 3, 8% of *Nicotiana. tobacum* and between 7-12% plant of *Nicotiana rustica*.

1.6: Prevalence of Smokeless - Tobacco in the World:-

The practices and the prevalence of smokeless, spit tobacco are significantly high in Africa and the Middle East. These products pose serous negative health consequences. Snuff, toombak, shammah (brands, bejeli ,haradi sharaci ,black shammah), commercially packaged chewing tobacco (brands, gudkha and pan masala) are several smokeless tobacco (ST) products available for oral use either (Dipping or Chewing) or nasal use in almost parts of Africa and some parts of middle Eastern countries. Millions of people use it. The dry fermented products are especially consumed in Arab countries of North Africa, including Libya, Tunisia, and Algiers. In West Africa Malawi, Cameroun, Ghana and Nigeria. Snuff product, chicambo, chic and taba respectively are consumed. Toombak is a snuff product particularly, used by more than 10 millions population of the Sudan and neighboring countries. Arabia shammah is prevalent in Yemen

and southern west parts of Kingdom of Saudi Arabia in Gizan province (Idris, 1992).

There are primarily four smokeless tobacco products: loose leaf or chewing tobacco, snuff, plug tobacco and twist or roll tobacco. Chewing tobacco and snuff are the most widely distributed in the world?

Originally snuff was used for nasal application (sniffing) in some parts of the world e.g. in Bavaria, Germany and in South Africa. Sniffing is still practiced to day. However, snuff is now customarily used orally by placing it between lower gum and cheek or lip (Dipping). In North America and Western Europe, snuff is manufactured from black tobacco varieties of *Nicotiana glauca* by curing, fermentation and aging. Also it is most often used orally as finely ground tobacco powder (IARC, 1986 and USHHS, 1986).

Cigarette smoking is pandemic affecting large proportions of the population world wide in contrast the use of smokeless tobacco is endemic ,largely restricted to certain geographical areas such as North America, the Scandinavian countries, India ,Bangladesh, South east Asia and parts of Africa. Of the commercially available forms of smokeless tobacco ,snuff is probably the most wide spread it constitutes pulverized tobacco that is most often moist and taken orally ,either in loose form or in tea bag -like small packages (sachets) placed between the gum and the chin or under the upper lip (Asplund ,2001).

The use of smokeless tobacco defined as either snuff or chewing tobacco ,is a popular habit in United states with an estimated six million regular users (Creath *et al.*, 1988), and the habitual use of chewing tobacco

is rising in the United states (Everett *et al.*, 1998). Tobacco in its various forms has been used for centuries in Pakistan. Tobacco is used both in smoking as well as in smokeless form but smoking cigarettes is common throughout the country, nasswae is pan atype of smokeless tobacco popularin Karach and some cities of Punjab, which is a powdered tobacco mixed with ash or lime and some flavoring or coloring agents is placed between the gum and the lower lip, this type of smokeless tobacco is very popular in Baluchishtan and north west Frontier province (McMichael , 1984 ;Vogler *et al.*, 1962) Smokeless tobacco use is practiced in many forms. Chewing of tobacco containing products or snuff –dipping habits vary greatly form one part of the world to another. While consumption of (ST) as oral snuff is prevalent in the USA (National institutes of health, 1989) and in Scandinavian countries (Axell, 1993). Loose tobacco is often added to betel quid in south Asia (IARC, 1985).

The use of snuff is common world wide, but more common in the southern parts of the united stats, the Scandinavian countries southern parts of the Kingdom of Saudi Arabia, southern Africa countries and in the Sudan in northeast Africa. Scandinavian snuff locally called snus, has been used for centuries in Denmark. The habit of snus dipping is widely prevalent and the quid is usually placed between the lower lip and the alveolar (Pindborg *et al.*,1962).In Norway the habit of snus dipping is uncommon , but presently is increasing particularly among young individuals and the quid is placed under the upper lip (Schei *et al.*, 1990;Strom *et al.*, 1998).

In Sweden, the habit of snus dipping is the eldest and the date back to the year 1637. In Sweden, snus consumption declined for several decades

during the period 1920-69 (Andersson, 1991), and increased by 92% during the period 1970–92., and currently is the only tobacco product with increasing sale. Snus product for oral use is moist with a pH value in the range of (7.8 -8.5) and many different brands are commercially available. However the majority of snus, users prefer only one or two brands. The most popular way of practicing the habit is by placing the quid in the upper gingivolabial sulcus. Sweden has the highest per capita consumption and sale of snuff in the world. (Andersson,. 1991 and Hirsch, 1983).

1.7. Cultivation of toombak in Sudan:-

Toombak grows in silky or sandy soil which receives heavier rainfalls in the North West of the Sudan, after end of the raining season September/ October toombak is planted during the months November / December and never irrigated. At first it is broadcasted in the farm and then transferred to new areas which are called (Makhamas). Harvesting starts in the months February/March when the leaves turn yellow and brownish spots start appearing (called the small pox stage). Harvested leaves are left in the field for uniform drying, tied into bundles, moistened with sprinkling of water and stored for fermentation for couple of weeks at temperature ranging from 30 to 45°C during which bundles are separated for uniform drying during the months April/May. Tobacco leaves are ground and stored for a year for ageing (Idris, 1992).

1.8. Processing of Toombak:-

Tobacco leaves after cutting the trees are dried in a big basket fermented and the color changes from yellow to brown after the fermentation process. The leaves were then milled using electrical miller.

The product is milled to different particles size this is mainly related to consumer taste consideration. Since in Eastern part of Sudan people prefer the coarse product while in Khartoum and central region, they prefer the fine or powdered product. The milling process is done in the same areas of cultivation in Sudan. Most of milling machines are centered in El fashir town in Darfour province.

Processing of toombak for sale is usually carried out manually in toombak shops by toombak vendors. It is performed by preparing four parts of a coarse powder of dried toombak leaves in a bowl and in another the concentrate of sodium bicarbonate is added gradually in small amounts to the tobacco (Idris, 1992). While adding the solution, the product is mixed vigorously by both hands (Fig 21), and concurrently tested by sensation of the fingers tips until it becomes moist and hardened. The output is then transferred to special air tight tin containers which are then covered firmly for about 2 hour thereafter the product becomes ready for sale or use. Before buying users generally ask for a bit to smell or test, since the aroma and test decide the quality rank of the product. Currently, toombak is sold in small plastic bags each taking about 100g. Some toombak users carry round or box shaped tin cans in his pocket named hookah and is similar to plastic bags though some people use king size. Hookah is still used by some people and it make an indentation in the pocket of user, thus one can easily

guess and identify. This helps users to communicate with each other for exchange of brand or request of a dip. A regular user consumes an average of 10- 20 dip per day, thus requiring a steady supply of toombak.

1.9. The measurement of pH, Moisture and Nicotine in toombak:-

The moist toombak, with strong aroma highly addictive and it is used widespread particularly among males greater than women. It has pH range between (8-11), moisture content ranges 6-60% and nicotine content is from 8 to 102 mg/ g wt. (Idris *et al.*, 1998). The addition of natron which has a pH value of 9.0 raised the pH degree of the fine and coarse toombak.

1.10. Natron (Sodium bicarbonate):-

Natron or atron is also called sodium hydrogen carbonate. It is a mineral rock with the chemical formula $\text{Na}_2\text{H}(\text{CO}_3)_2 \cdot 2\text{H}_2\text{O}$. Its colour is grey to yellowish white and is of alkaline pH. There is no information on either the history or reasons behind use of atron as an additive to toombak. It may be used to homogenize the leaves to a fine sticky form as atron is used in the Sudan to homogenize vegetables during cooking. Atron, opposed to lime in other part of the world, is probably added to toombak for its alkaline effects. It has been shown that at high pH (11.0-11.8), nicotine is completely protonated and its rate of absorption is increased (Brunnemann *et al.*, 1974).

Studies of nass, a type of snuff used in the former USSR which contains lime and has high pH (11-11.8), has shown that when the product is placed in the mouth, nicotine reaches the central nervous system very quickly. Thus pH value in tobacco products can influence the absorption

and thereby the extent of pharmacological activity of nicotine (Brunnemann *et al.*, 1995).

1.11. The habit of toombak in the Sudan:-

Toombak can be bought from innumerable shops in the market, and the product is advertised extensively at points of sale where vendors tend to use commercial names to attract buyers. The habit of toombak dipping is practiced by taking a small portion from the bag or hookah with the three fore-fingers, usually of the right hand, putting it in the palm of the left hand, and manipulating it by the thumb and middle fingers of the right hand until it forms a ball called (Saffa) which is of about 10g in weight. The Saffa is not chewed but dipped and retained between gum and lip or cheeks or floor of mouth, and sucked slowly for about 10-15 minutes. Generally, men prefer dipping between the lower lip and gum, while women prefer dipping between cheeks and gum. The dipping continues for a period ranging from a few minutes to several hours, until the Saffa becomes bland. Men periodically spit the insoluble debris that is freed from the bulbous and the saliva which is secreted during toombak use, whereas women retain the Saffa without spitting because of social unacceptability. The mouth is usually rinsed with water after the quid is removed. The toombak quid is sometimes retained in the mouth during sleep (Idris, 1992).

1.12. Socio-Economical Background about Toombak in Sudan:-

Toombak played an essential role in socio-economic life of some Sudanese people. It is considered as an important product in many areas in Sudan mainly in the Western State. Many surveys have been conducted that the production and marketing of toombak was very profitability to the

farmers and the merchants together. In Northern Darfour which is the major provinces where toombak is cultivated, an where all people are involved in this activity, they found that toombak is the main cash crop in this region and constitute about 80% of the gross domestic products. The marketing unit of toombak is quntor, on the other hand the commerce of toombak supported government by tax as Gebana and customs. In addition some Sudanese people export the toombak to neighboring countries and sell it with high prices.

1.13. Absorption of Nicotine in the Body:-

Nicotine absorption occurring at different parts of body chiefly in the mucosal tissue of mouth, respiratory tract, intestine and skin (Hussain, 1984).

There are a few studies that have directly examined the effects of pH on nicotine absorption , Beckett *et al.*, (1972) found very little buccal absorption of nicotine from tobacco when the pH was 5.5.Ten percent absorption at pH of 7, and about 30% at pH of 9.0. Henning field *et al.*, (1990) found that rinsing with acidic beverages such as coffee or cola before chewing nicotine polacrilex nearly eliminated nicotine absorption. These results indicate that pH is an important determinant of buccal absorption of nicotine. Benowitz *et al.*, (1988) compared nicotine absorption from a moist snuff to that from cigarette smoking and nicotine gum. The authors estimated absorbed doses of nicotine to be twice as high for moist snuff compared with smoking (1.8, 3.6 mg for smoking and snuff, respectively).

The nicotine-dosing potential of moist snuff is determined by at least three factors: The amount of nicotine in the product, the pH level of the product, and the size of the tobacco cutting.

Henning field *et al.*, (1995) found that the nicotine content of six moist snuff products ranged from (7.5mg/g to 11.4mg/g) and that the pH of these products ranged from (6.9 to 8.6). The pH of the snuff is important because nicotine most readily crosses the oral mucosa in the unionized form. The degree to which nicotine is unionized depends on the higher pH levels (more alkaline).

The rate of absorption is highest when the snuff is first placed in the mouth and plasma concentration continued to rise until the snuff was removed from the mouth. Absorption continued even after the snuff was removed, presumably because of the slow release of nicotine from the mucosa into the plasma or absorption of swallowed nicotine in the gut.

1.14. Metabolism of Nicotine in the Body:-

When nicotine is absorbed, immediately distributed into different parts of the body brain, lungs, liver, intestine, spinal cord and adrenal gland (Hussain, 1984). Liver is the site of breaking down of nicotine into harmless compounds which passed in urine with small amount of unmetabolized nicotine.

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1.15. Physiological and Pharmacological effects of nicotine in the

Body:-

Large amounts of nicotine are delivered rapidly to the blood stream during use of moist snuff. In fact venous nicotine concentrations are higher than those which have been observed following cigarette smoking.

Benowitz *et al.*, (1988) found that average peak blood nicotine concentration increased 14.3ng/ml after smoking one cigarette or using 2.5g moist snuff for 30 minutes. In a study of four brands of moist snuff were tested have comparable nicotine content (11.4, 10.4 and 11.4mg/g respectively), but produced different pH values in suspension (8.6, 7.6, and 7.5 respectively (Henning field *et al.*, 1995), these study confirm that the pH of these products in suspension is a significant factor in determining nicotine bioavailability and increasing of heart rate after moist snuff administration is associated with the nicotine levels attained by each product. The heart rate increases during the first 15 minutes of administration and then declined after about 15 minutes of administration and despite continued increases in nicotine plasma concentration. This fact also shown by (Benowitz *et al.*, 1988).

In addition the high level of nicotine in blood stream produces nausea, vomiting, cardiovascular diseases and other health hazards associated with the use of moist snuff are poorly documented (Asplund, 2001), but three studies performed in Sudan have consistently demonstrated a much lower risk of myocardial infraction and sudden death among snuff users than among cigarette smokers (Huhtasaari *et al.*, 1992; Huhtassari, 1999). In study (1) where as no excess risk atall for myocardial

infraction was observed in snuff users but in (2) and third study showed a 40% higher risk of cardiovascular death in snuff dippers compared with non users of tobacco (Bolinder *et al.*, 1994).

Nothing is known about the influence of smokeless tobacco on risk of stroke, but the presence of the tobacco-specific nitrosamine and polycyclic aromatic hydrocarbons one candidate for etiological agents for cardiovascular disease (Hecht *et al.*, 1993; Benowitz *et al.*, 1997). These substances can be absorbed to a considerable extent from snuff (Hecht *et al.*, 1993).

Exposure to high concentrations of nicotine has adverse effects on a number of physiological and biochemical processes involved in atherosclerosis (Kilaru *et al.*, 2001). Two studies that have used ultrasound to investigate thickness and other signs of atherosclerosis in the carotid arteries have observed in regular snuff dippers, in contrast to smokers; do not have more carotid arterial disease than non users of tobacco (Bolinder *et al.*, 1997; Wallenfeld *et al.*, 2001).

The use of snuff is now more common among men than smoking, has the lowest prevalence of smoking in Europe (and the lowest lung cancer rates) (Kuulasmaa *et al.*, 2000). On the other hand there is often heavy addiction to nicotine among snuff dippers.

In addition, there is evidence that smokeless tobacco use may increase the risk of cardiovascular disease and cancers of the larynx, esophagus, and other sites, as well as disease of gingival and periodontal tissue (USDHHS, 1986). Recent data suggest that some forms of smokeless tobacco may increase the risk of dental caries (Tomar *et al.*, 1998).

The increased popularity of toombak use in recent years seems to be due to it satisfying some psychosocial, pharmacological economical and social demands.

Regarding the psychosocial demands, toombak helps to alter mood, and ambiguously helps both concentration and relaxation and distraction. That is provided by both the intervals of preparation of the saffa and the dipping. The pharmacological effects are mainly attributed to nicotine that is a powerful pharmacological agent that changes the cardiovascular, neural, endocrine, and muscle function and induces effects in the gastrointestinal tract (The health consequences of using smokeless tobacco, 1996).

The cardiovascular changes include increased hear rate, blood Pressure and decrease in skin temperature due to vasoconstriction in the extremities. The nervous effects in the brain and the peripheral nervous system are associated with changes in electrical cortical activity, i.e: induction of both stimulation and relaxation. In the gastrointestinal tract, nicotine stimulates the parasympathetic autonomic ganglia and brain stem, causing the release of pharmacologically active substances which may produce nausea, vomiting and occasionally diarrhea, therefore, it is now accepted that tobacco causes physical dependence addiction and habituation (The health consequences of using smokeless tobacco, 1996).

1.16. Nitrosation of Nicotine:-

Natural tobacco contains at least 3050 different compounds (Roberts, 1988). Furthermore, smokeless tobacco may be enhanced by flavoring agents, added in the form of plant extracts and /or as chemicals (Roberts, 1988, Mookerjee *et al.*, 1988).

Among 23 tumorigenic agents in smokeless tobacco (Wynder *et al.*, 1967; Sharma *et al.*, 1985), are volatile aldehydes and N-nitrosamines, Nitrosamine acids, lactones, poly nuclear aromatic hydrocarbons pyrene, primarily benzo, and carbomates, certain metals and the emitters, polonium-210 and uranium -235 and -238.

The most abundant strong carcinogenic compounds in smokeless tobacco are the tobacco-specific N-nitrosamines (TSNA).these are formed by N-nitrosation of the major habituating tobacco alkaloid nicotine, and of minor nicotiana alkaloids during tobacco harvesting, curing fermentation and ageing.Seven TSNA have been identified in smokeless tobacco (Djordjevic *et al.*, 1989; Hecht *et al.*, 1988). Of these N-nitrosonornicotine (NNN) and 4- (methylnitrosamine) -1-(3-pyridyl)-1-butanone (NNK) are the predominant carcinogens in smokeless tobaccos. Sudanese toombak contains high concentration of TSNA, due to the use of *N. rustica* in its preparation. NNN and NNK levels in *N. rustica* have been reported to be much higher than *N. tobaccum* (Bhide *et al.*, 1987).

1.16.1. N-nitrosamines:-

Using gas chromatography with thermal energy analysis, the TSNA types; N- nitrosonornicotine (NNN), 4-(methylnitrosamine)-1-(3 pyridyl)-1-

butanone (NNK), N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB) were quantified in toombak and saliva of toombak users (Idris *et al.*, 1991, Idris *et al.*, 1992). Exceptionally high level mean; range, mg/g toombak dry wt) of NNN (1.13; 0.50-3.08), NNK(2,31; 0.62-7,87;31), NAT(0.08;0,02-0.2) and NAB(0.22;0.02-2.37).

Two additional TSNAs, 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanol (NNAL) and 4-(methylnitrosamine)-4-(3-pyridyl)-1-(butanol (iso-NNAL) were found in the saliva of toombak users for the first time and were confirmed by gas chromatography mass spectrum (Idris *et al.*, 1992).

The most abundant carcinogens in smokeless are the tobacco – specific nitrosamine, two of which , N-nitrosornicotine (NNN) and 4-(methyl-nitrosamine)-1-3-pyridinyl)-1-butanone (NNK), have shown to increase longevity of mucosal cell in vitro with growth and morphologic changes suggestive of cell transformation (Murrah *et al.*, 1993), and to induce benign and malignant tumors at various sites in mice ,rats and hamsters (Hecht *et al.*, 1986; Hoffmann *et al.*, 1991).

Both NNN and NNK have been shown to pyridyloxobutylate DNA and NNK has also been shown to methylate DNA (Murphy *et al.*, 1990).

In a study to compare the concentrations a level of TSNAs in snus and toombak cleans that toombak contains concentration of TSNAs in 100 fold higher than those found in snus. The level of NNN and NNK in the saliva of toombak dippers is also significantly higher than those found in the saliva of snus dippers. It has been estimated that more than 80% of the carcinogenic TSNAs are extracted from the toombak by saliva and negative pressure during sucking of dipped toombak quid (Prokopczyk *et al.*, 1992).

Tobacco smokers, chewers of tobacco and betel quid (often containing tobacco) and snuff dippers are all exposed to increased levels of nitrosamine precursors (such as nicotine and/or arecoline, nitrate, and nitrite No_x) and nitrosation modifiers (thiocyanate aldehydes), (Hoffmann *et al.*, 1986; Hecht *et al.*, 1988). Hoffmann and Hecht (1988) have deduced that TSNA in take through life-long use of tobacco is one possible explanation for tumor induction in human.

Tobacco smoking, chewing, and snuff dipping, factors contributing to the endogenous formation of N-nitroso compounds (Noc) which increase the risk of tumorigenesis beyond that incurred by the body's load of ingested and/or inhaled carcinogens. A sensitive procedure for estimating human exposure to endogenously formed (Noc) was developed by Ohshima and Bartsch (1981) their method is based on the quantities excretion of non carcinogenic N-nitrosoproline (NPRO) and other N-nitrosamine acids (NAA) in the urine which are measured as an index of endogenous nitrosation after ingestion of precursors (proline and/or nitrate) with and without ascorbic acid. Human urine contains several NAA, the main ones being NPRO –N-nitrososarcosine (NSAR), N-nitrosothiazolidine -4- carboxylic acid (N-nitrosothiopline, NTPRO), and the trans isomers of N-nitroso-2- methythiazolidine -4- carboxylic acid (N-nitroso-2-methythiopline, NMTPRO). Ohshima *et al.*, 1984. Tsuda *et al.*, 1987, Ohshima *et al.*, 1983, Tsuda *et al.*, 1984).

This method is to be used to determine the amount of nitrate added to whole tobacco as antimicrobial agent and does not distinguish between the

amounts of added and the amount of naturally occurring nitrate found in whole tobacco.

1.17. Disease association to smokeless-tobacco use:-

1.17.1. Cancer:-

When investigating intra-oral cancers of sites indirect contact with tissue is an important to these with little or no contact, confirms the hypothesis that direct contact with tissue is an important factor in tobacco carcinogenesis in the mouth (Idris *et al.*, 1995).

Oral use of snuff has been associated with increased risk for oral cancer in Western Europe and North America (IARC, 1985 and USDHHS, 1986). Chewing of tobacco has also been associated with increased risk in South and South East Asia (IARC, 1985). Toombak is a form of oral snuff extensively used in the Sudan (Idris *et al.*, 1995). This form of snuff has been shown to contain unusual levels of the carcinogenic tobacco specific Nitrosamines (Idris *et al.*, 1992). The highest relative frequency of oral cancer in Africa has been reported from the Sudan (Hickey 1959; Idris *et al.*, 1995; parkin, 1985).

Although a high relative frequency of oral cancer in the Sudan has been observed since the years 1959 and 1963 (Hickey 1959; Lynch *et al.*, 1963). The earliest observation on the association between oral cancer and use of toombak was reported in 1980 (Idris *et al.*, 1980). Much later; it was found that 81% of patients with oral squamous cell carcinoma (SCC) from the Sudan used toombak (El besheir *et al.*, 1989).

Recently, abundant information on toombak use and development of oral cancer has been reported, and the cancer was frequently found at the site where the toombak quid is placed (Idris *et al.*, 1991; Idris *et al.*, 1994). Studies of a similar nature from Saudi Arabia have suggested the etiological oral of Shammah for development of oral cancer (Salem *et al.*, 1984; Stirling *et al.*, 1981). Snus dipping in Sweden apparently increases the risk of oral cancer at the site where snuff is placed (Axell *et al.*, 1978). Prevalence of oral cancer is high in Sudan and the disease is attributed to N- introsamine rich oral snuff consumption (Hussain *et al.*, 2003). Oral precancerous / premalignant lesions have been shown to precede the development of oral squamous cell carcinomas OSCCs.

The habits of tobacco use and alcohol consumption have been strongly attributed to the development of these lesions (Johnson *et al.*, 1993, Franceschi *et al.*, 2000) however; in Sudan alcohol conception is uncommon, while use of tobacco in the form of snuff is common.

A close relationship between the use of toombak and development of OSCCs has been reported. The relative frequency of OSCCs in respect to all body malignant (12, 6 %) was previously reported from Sudan (Idris *et al.*, 1995). Eighty percent of these malignancies are attributed to the use of toombak.

The use of exfoliative cytology for diagnosis or screening of oral mucosal lesions has received considerable and it was found reliable in the diagnosis of oral hairy leukoplakia (Frago *et al.*, 1992) and during follow up of patients with a history of head and neck cancer (Pellanda, *et al.*, 1999). Further exfoliative cytology has been described a useful method for

detecting oral premalignant and malignant lesions (Ramaesh, 1998).this method act as a source of useful markers of field cancerization like cytokeration 16 cytokeratin 19 and histo-blood group antigen H type 2 chain that might be applied to monitor and /or predict the occurrence of second primary tumors in patients with head and neck cancer. Recently it has also been shown that cytological smears can provide an adequate quantity and good quality of DNA that is useful for restriction site atypia.

Epidemiological evidence suggests that toombak is a risk factor for cancer of the oral cavity and possibly of the oesophagus in the Sudan (Babekir *et al.*, 1989).

One study published in 1981 provided strong evidence of a strong relationship between snuff use and oral pharynx cancer among women in North Carolina (Winn *et al.*, 1981).

Use of smokeless tobacco does not increase the risk of cancer in digestive organs .Alcohol emerges as a major risk factor for cancer of digestive organs and oral cancer with strong does -response relationship between the amount of drinking and risk.

Smoking associated with increased risk of oral cancer but not of cancer of the digestive organs (Thequdor *et al.*, 1992). Oral snuff application induces bening malignant tumors of the palate, gingival and tongue (Hecht *et al.*, 1986, Jhansson *et al.*, 1989).

1.17.2 Oral mucosal lesions found in smokeless tobacco users:-

Use of smokeless tobacco (chewing tobacco and snuff) has been associate with mucosal lesions (Axell *et al.*,1976 ; Wolfe *et al.*,1987), and

some of these lesions may eventually become malignant (Roed *et al.*, 1973;Mehta *et al.*, 1981).Oral mucosal lesions occur at the site of placement of the tobacco (Greer, 1983; Hirsch, 1982 ; Poulson, 1984). And preliminary evidence suggests that lesions are associated with duration and amount of smokeless tobacco use. Clinical and histopathological characteristics of toombak - associated oral mucosal lesions detected in an epidemiological study in northern Sudan in 1992/1993, are described the lesions site in the majority of toombak users was the anterior lower labial groove and the lower labial mucosa. Four degrees of clinical severity of lesions, similar to those used to characterize Swedish snuff dipper's lesions. Were applied an association between the severity of mucosal lesions and a longer life time duration (>10 years) of toombak use was found, but the severity was not related to daily frequency of the habit. Parakeratosis, pale surface staining of epithelium and basal cell hyperplasia were commonly observed, but epithelial dysplasia was infrequent (Idris *et al.*, 1996).

A possible association of toombak use with oral squamous carcinoma has been reported (El Beshir *et al.*, 1989; Idris *et al.*, 1995) but whether such carcinomas are preceded by keratotic or dysplastic lesion is unknown.

A decreased vitamin C intake also was found among the ST users. The ST users with epithelial dysplasia as compared with those with hyperkeratotic lesions were slightly older had a lower intake of vitamin C.

A physiological disorder of the tobacco plant, termed frecking. Although the etiology of this condition is uncertain, so called organic toxins produced by bacillus species found in the soil where tobacco plant is grown have implicated (Tso,T.C. 1972). It is conceivable that bacillus spores could

contaminate chewing tobacco processed for human use and germinate when placed on to the oral mucosa, the bacteria could then elaborate potent virulence factor, such as proteinases, that activate oral keratinocytes and the kallikrein / kinin metabolic pathway in the oral mucosa, leading to plasma exudation and tissue injury (Imamura, *et al.*, 1994 ;Rubinstein *et al.* , 2001).

1.18. Mutation of the p53 gene:-

The p53 tumour suppressor gene encodes a nuclear- phospho-protein that plays an important role in cell proliferation and differentiation (Harris, 1990; Nigro *et al.*, 1989). The most common genetic alterations identified in human cancers are mutations in the p53 tumour suppressor gene .A high frequency of p53 mutations have been observed in tobacco –related cancers (Greenblatt *et al.*, 1994).

To determine if toombak use may be associated with the induction of mutations in the p53 tumor suppressor gene we screened four head and neck squamous cell carcinomas SCCs obtained from three toombak-using patients and one from non toombak-using patient. Using polymerase chain reaction PCR/single-stranded conformational polymorphism SSCP analysis and DNA sequencing. P53 mutations were found in tumors resected from two of three toombak-using patients one at codon 282 (CGG-TGG) and the other in intron 6 (AT-GC). No p53 mutations were observed in the tumor from the non-toombak-using patient. (Philip Lazarus *et al.*, 1996).

Salah *et al.*, (1999) using PCR-SSCP/DNA sequencing methods observed significant increased incidence in mutations of p53 gene in oral squamous cell carcinomas OSCCs from toombak dippers (93%) as compared with those from non-dippers (57%) in Sudan. In Scandinavia

(61%). In OSCCs from dippers, mutations were found in exons 5-9, while in those from non-dippers they were found in 5,7,8,9 and no mutations were found in exon 8 in any of the OSCCs from Sudan. Certain types of mutations however were similar with respect to exposure to toombak. OSCCs from dippers showed 15 transversions, 9 transitions, 3 insertions and one deletion, compared with 7 transversions, 2 insertions and one deletion found in OSCCs from Sudanese non-dippers, and 9 transversions, 17 transitions and 2 insertions found in those from non-dippers in Scandinavia. No mutations were found in any of the non-malignant oral lesions in relation to dipping or non dipping status. These findings suggest that the use of toombak plays a significant role in induction of increased p53 gene mutations. Mutations observed were such similar to those induced by tobacco –specific N-nitrosamines TSNA. In experimental animal models and those already reported in toombak dippers, in OSCCs 98% of p53 gene mutations have been described in exons 5 to 8 in codons 238- 248 exon 7 and 278- 281 exon 8 and these DNA regions are likely hot spot areas (Raybaud *et al.*, 1996).

1.19. Smokeless –tobacco use induce some viruses:-

Smokeless tobacco contains chemical tumor promoters that promote induction of latent Epstein-Barr virus (EBV) to active replication for example N-nitrosornicotine (NNN), (NNK) and benzo (a)pyrene (Bap) (Hal *et al.*, 1999). This virus is associated with certain carcinomas including nasopharyngeal carcinoma, lymphoma (Raab and Traub, 1996).

And smokeless tobacco –specific nitrosamines interact with herpes simplex virus and demonstrate synergism for cell transformations, supporting a possible synergistic relationship for oral carcinogenesis between herpes simplex virus and smokeless tobacco (Park *et al.*, 1991; Murrah *et al.*, 1996).

Also other human papilloma virus (HPV) genomes were more often encountered in leukoplakias associated with smokeless tobacco use ,showing among 100 instances of benign leukoplakia ,only 4% of non- tobacco related and 10% of smokeless tobacco- related lesions harbored viral sequences (Robert et al ., 1990).

HPV is an infections agent that increasingly associated with mucosal cancer and associated with genital cutaneous warts, but is not common in oral premalignant and malignant neoplasms.

Expression of keratins (Ks) is one possible marker of malignant potential.In the epithelium of uterine cervix, presence of human papilloma viruses (HPVs)(salah *et al.*,1998).There is a high level of expression of K13,K14 and K19 in oral carcinomas from snuff dippers compared to those from non snuff dippers (Salah *et al.*,1998).

Keratins (Ks) are a group of insoluble portions that make up a family of 20 poly peptide in human, and are subject to differential expression (Moll *et al.*, 1990).They constitute two types: acidic, or type one which includes K9 to K20 and basic or type two which includes K1 to K8 (Clousen *et al.*, 1986).Of the acidic type, K13 is known as a differentiation marker for non-keratinizing epithelium, while K14 is constitutionally expressed by all stratified quamous epithelium. K19 is present as a major

component in simple epithelia, and may be present as a minor component in stratified squamous epithelium. In the oral mucosa distribution of these Ks has been found to reflect the differentiation pattern of particular intraoral sites affected by pathological processes (Moll *et al.*, 1990).

Expression of these Ks as differentiation markers in oral cancer and precancerous lesions from western populations has been studied (Moll *et al.*, 1990). To our knowledge, no studies have examined the expression of any type of Ks in oral mucosal lesions from African populations.

In western countries, many studies have identified human papillomavirus (HPV) antigens and viral DNA in potentially malignant and malignant oral lesions. Out of the 77 or more different (HIV) types more than 12 have been found in these lesions, including 1, 2, 4, 6, 7, 11, 13, 16, 18, 30, 32, and 57 (Shijders *et al.*, 1994). Nevertheless the prevalence described ranges from 0-80% (Sugerman *et al.*, 1997). Studies on HPV infection from African populations are limited. (Padayachee *et al.*, 1995; Williamson *et al.*, 1991), suggesting that HPV is possibly not an etiological factor for development of oral cancer (Van-Rensburg *et al.*, 1996).

HPVs have been shown to disturb the keratinocyte differentiation in the base cell layer of the oral epithelium, with the suggestion that K19 staining may help an understanding of the interaction that may possibly occur between virus and host cell (Chang *et al.*, 1990).

Additionally, expression of K19 at high levels has been reported in virally and chemically transformed human epidermal keratinocytes (Banks-Schlege *et al.*, 1986).

Expression of Ks in carcinomas form snuff dippers perhaps arises from dysregulation of keratinocytes, proliferation, and maturation. Probably induced by the physical action caused by continuous all day application of snuff on the oral mucosa, or by the chemical action of the TSNA's thought to be involved in the development of these cancers (Idris et al., 1991).

1.20 Contamination:-

Growing plants carry atypical flora of microorganisms on their surfaces and may become contaminated from outside sources such as soil dust which is whipped up by air current. Nearly all microorganisms are important in foot microbiology for example molds, yeast and species of bacteria genera such as *Bacillus*, *Clostridia*, *Enterobacteria*, *Escherichia*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus* and *Acetobacter* (Frazier and Westhoff, 1978).

1.20.1 Bacteria associate with smokeless tobacco use:-

Five *Bacillus* species, predominantly *Bacillus megaterium* (eight isolates) *B. pumilus* (six isolates), five isolates of *B. brevis*, two isolates of *B. licheniformis* and one isolate of *B. subtilis* were isolated from two popular brands of commercially available chewing tobacco in United States (Israel and Gerald, 2002).

1.20.2 Bacillus species:-

Bacillus species represents a genus of gram-positive bacteria which are ubiquitous in nature (soil, water, air borne dust) and some species are normal flora in human intestines. The ubiquity of *Bacillus* species in nature

due to the unusual resistance of their endospores to chemical and physical agents ,the developmental cycle of endospores formation ,the production of antibiotics ,the toxicity of their spores and protein crystals for many insects.

1.20.2.1 Classification of Bacillus species:-

In Bergey's manual of systematic Bacteriology (1st Ed 1986) the family Bacillaceae features six genera of endospore – forming bacteria. *Bacillus* is distinguished from the other endospore- forming bacteria on the basis being a strict or facultative aerobe, rod shaped, and usually catalase-positive.

Other endospore-forming genera in this family are spore *Lactobacillus*, which is microaerophilic and catalase-negative *Clostridium*, which is anaerobic and does not reduce sulfate, *Desulfotomaculum* ,which is anaerobic but does reduce sulfate, *Sporosarcina*,which has acoccal morphology, and *Thermoactinomyces* ,which while forming endospores ,displays typical actinomycete characteristics. These genera are related phenotypically as gram positive bacteria that form endospores and they are not related phylogenetically.

There are 40 recognized species in the genus *Bacillus* listed in Bergey's manual of systematic Bacteriology (vol 2 1986) in addition to more than 200 species of genus *Bacillus* have been described after 1986.

In Bergey's manual of systematic Bacteriology (2nd-ed 2001) phylogenetic Classification of endospore– forming bacteria landed all of the genera named above in two different classes of gram – positive, *Clostridia* and *Bacillus*. *Clostridium* include in the order *Clostridiales* and the family

clostridiaceae, while *Bacillus* include in the order bacillale and the family bacillaceae.

Early attempts at classification of *Bacillus* species were based on two characteristics: aerobic growth and endospore formation. This resulted in tethering together. Many bacteria possessing different kinds of physiology and occupying a variety of habitats. Hence, the heterogeneity in physiology, ecology, and genetics, makes it difficult to categorize the genus *bacillus* or make generalization about it. There is great diversity in physiology among members of the genus, whose collective features include degradation of most substrates derived from plant and animal sources including cellulose, starch, proteins, agar, hydrocarbons, and the others, antibiotic production, nitrification, denitification, nitrogen fixation, facultative lithotrophy, autotrophy, acidophily, alkaliphily psychrophily, thermophily and praassitism, sporeformation, universally found in the genus, is thought to be strategy for survival in the soil environment ,where in the bacteria predominate aerial distribution of the dormant spores probably explains the occurrence of *bacillus* species in most habitats examined.

Many criteria have been proposed as basis of classification of *Bacillus* species such as the G-C content of *bacillus* species which ranging form (32 to 69%). This observation reveals the heterogeneity of genus, not only is there variation from species to species but there are some times profound differences in G-C content within strains of a species .For example the G-C content of *B.megaterium* group ranges from (36 to 45%).

Another approach to *bacillus* taxonomy has been analysis of 16s rRAN molecules by oligonucleatides sequencing. This technique of course

also reveals phylogenetic relationship surprisingly, *Bacillus* species showed akin ship with certain non spore forming spp including *Planococcus* and *Lactobacillus* (Kenneth, 2005).

1.20.2.2 Endospores:-

Endospores were first described by Cohn in *Bacillus.subtilis* and later by Koch in pathogen, *Bacillus.anthraxis*.Cohn demonstrated the heat resistance of endospores in *Bacillus.subtilis* and Koch described the developmental cycle of spores formation in *Bacillus.anthraxis*, although they are eventually released from mother cell or sporangium as free spores.

Endospores have proven to be the most durable type of cell found in nature, and in their crypto biotic state of dormant, they can remain viable for extremely long period of time ,perhaps millions years (Kenneth, 2005).

1.20.2.3 Pathogenicity of Bacillus species:-

Although most species of *Bacillus* are harmless saprophytes, two species are considered medically significant *B.anthraxis* and *B.cereus*

B.anthraxis is the bacterium, which causes anthrax in most animals: cattles, sheep, camles and some times human, and the infection is classified as one of three types: cuaneous infection (95%of human cases), inhalation anthrax (rare) and gastrointestinal anthrax (very rare).

B.cereus unlike *B.anthraxis* is a motile bacterium which can cause toxin-mediated food poisoning. It is known to inhabit many kinds of food including stew, cereal, and milk most recently; however, it has been found in fried rice.

Two toxins are responsible for clinical illness an emetic toxin that causes vomiting and enterotoxin that associated with diarrhea. The emetic or vomiting syndrome has been associated with a heat stable toxin of *B.cereus*, this syndrome clinically resemble staphylococcal food poisoning. Symptoms of nausea and vomiting usually develop 1 to 6 hours after ingesting food that contains the toxin. Patients usually recover about 6 to 24 hours after the onset of symptoms (Centers for Disease Control, 1986).

The diarrhea syndrome with symptoms of abdominal pain and watery diarrhea, caused by a heat labile toxin of *B.cereus*, clinically resembles *Clostridium.perfringens* food poisoning has an average incubation time of 10 to 12 hours. The flagellar (H) serotypes most commonly involved in the diarrheal syndrome are 1, 2, 6, 8, 10, 12 and 19 (Gilbert *et al.*, 1977).

Opportunistic infections by other *Bacillus* species however not all isolates are contaminants or harmless saprophytes. Serious opportunistic infections associated with significant morbidity and mortality, have been caused by variety of *Bacillus* species. The most frequently encountered species that causes opportunistic *Bacillus* infections is *B.cereus*. The invasive disease is not related to the food poisoning syndromes described previously. In addition to *B.cereus*, *Bacillus* species which may be clinically significant occasionally and viewed as potential opportunistic pathogens, include *B. subtilis*, *B. sphaericus*, *B. megaterium*, *B. pumilus*, *B. circulans*, *B. licheniformis*, *B. mycoides*, *B. macerans*, *B. coagulans* and *B. thuringiensis* (Turnbull *et al.*, 1991).

Drobniewski (1993) has defined six broad groups into which clinical infections caused by *B.cereus* (1) local infections particularly of burns,

traumatic or post surgical, and the eye (2) bacteremia and septicemia (3) central nervous system including meningitis, brain abscesses,(4) respiratory infections (5) endocarditis and pericarditis(6) and food poisoning including emetic and diarrheal syndromes.

The types of infection involving other *Bacillus* species have included septicemia endocarditis, osteomyelitis, myonecrosis, simulating clostridial gas gangrene, necrotizing bronchopneumonia, hecrotizing pneumonia, empyema meningitis peritonitis, and endphthalmitis.

1.20.3 *Providencia. rettgeri*:-

In 1904 Rettger provided the first description of an organism that eventually was isolated and recognized organism from chickens during an epidemic of fowl cholera, this isolate was unknown until 1918, when it was named *Bacterium.rettgeri*. Organism belonging to the genus *Providencia* has undergone many taxonomic changes since description, with frequent confusion and overlap between organisms of the closely related genus *Porteus* and *Morganella*. Synonym *bacterium rettgeri*, *Shigella rettgeri* (Hadley *et al.*, 1918; Weldin, 1927) *Proteus rettgeri* (Hadley *et al.*, 1918; Rustigian and stuart) *providencia rettgeri* (Hadley *et al.*, 1918; Brenner *et al.*, 1978).

Kauffman first proposed the genus name *Providencia* 1951, to day five *providencia* species are recognized: *Providencia stuartii*, *P.rettgeri*, *P. rustigianii*, *P.alcalifaciens*, *P.heimbachae*.

1.20.3.1 Pathophysiology of *Providencia rettgeri*:-

In human *Providencia* species have been isolated from urine, stool and throat, perineum and wound, *Providencia rettgeri* has been isolated from a number of animals including poultry. This species is pathogenic in animals and has been reported to cause meningitis in crocodiles. Urinary tract infection with *P.stuartii* is less common while *P.rettgeri* is associated with long urinary devices. Urinary tract infection with *P.stuartii* and *P.rettgeri* is typical signs and symptoms of dysuria, frequently cloudy urine, fever (Ebbing, 2002). *Providencia rettgeri* documented as a causative organism in association between urinary tract infections and hyperammonemia, which caused coma in child (Murray *et al.*, 2001).

Providencia rettgeri was reported to cause the purple urine bag syndrome (PUBS) in elderly woman (85 years old) living in nursing home and has long term urinary catheter. The culture and sensitivity results of supernatant clear purple urine reveals heavy growth of coliform species identified as *Providencia rettgeri* (Jubouri *et al.*, 2001).

CHAPTER TWO

Materials and Methods

2.1 Samples for Bacteriological Examination:

2.1.1 Types of Samples:-

Two types of samples were collected from different toombak shops, moist sample and dry sample without addition of natron solution.

2.1.2 Sources of samples:-

Samples were collected from different toombak shops in Khartoum, Omdurman and Khartoum North.

66 samples consisting of 50 moist samples (Fig19) and 16 of dry samples (Fig20).

2.1.3 Collection of Samples:-

Samples were collected in sterile containers and much care was taken to avoid contamination.

2.1.4 Preparation of Samples:-

In the laboratory, one gram of each sample were aseptically weighed in small pieces of sterile aluminum voyl, put into sterile tubes containing 5ml of normal saline. The mixture was shaken vigiously. A small portion of supernatant was inoculated with sterile loop into different types of solid media (Blood agar, Nutrient agar, MacConkey agar) and incubated at 37°C for 24-48 hr. Well isolated colonies of aerobic and anaerobic bacteria were subcultured for purification in fresh solid media.

2.2 Collection of the Blood:-

Blood for enrichment media was collected with sterile syringe by vein puncture of jugular vein of healthy donor sheep kept at the department for this purpose. It was defibrinated by adding suitable anticoagulant. Blood was then stored in 10ml volumes into universal bottles, stored at 4°C in till use for use in blood agar media.

2.3 Cultural Media:-

All those media were done according to the oxoid manual (1973) of culture media ingredients and Barrow and Feltham (1993) as follows:-

2.3.1. Solid Media:-

2.3.1.1. Blood agar (oxoid):-

Forty grams which consisted of 10gm peptone, 5gm NaCl, 15mg agar and 10 lab-lemco powders were suspended in 1 liter of distilled water and boiled to dissolve completely. The medium was sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.3. The medium was cooled to 45-50°C, then 7 of defibrinated blood was added, mixed with gentle rotation and poured aseptically into sterile Petri-dishes.

2.3.1.2. Nutrient agar (oxoid):-

Twenty eight grams which consisted of 2gm yeast extract, 5mg peptone, 5gm NaCl, 15mg agar and 1gm of lab-lemco powder were suspended in 1 liter of distilled water, boiled to dissolve completely, and sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.4. The medium was poured into sterile Petri-dishes or distributed in a 10ml amount into sterile screw-capped bottles and allowed to set in slope position.

2.3.1.3. MacConkey agar:-

Fifty two grams which consisted of 20gm peptone, 10gm lactose, and 5gm bile sold 5gm NaCl and 0.075gm neutral red in 1 liter of distilled water, were boiled to dissolve completely, and sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.4. The medium was poured in sterile Petri-dishes in 15 ml volumes.

2.3.1.4. Milk agar (oxoid):-

Twenty four grams which consisted of 3gm yeast extract, 5gm peptone, 15gm agar and 1gram of milk solids(equivalent to 10ml fresh milk) were suspend in 1 liter of distilled water, boiled to dissolve completely and sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.2, then distributed into sterile Petri-dishes.

2.3.1.5. Lecithovitellin agar egg yolk agar:-

Consisted of 100 ml of lecithovitellin solution (egg yolk emulsion) and 900ml of nutrient agar. The nutrient agar was melted and cooled to about 55°C before lecithovitellin solution was aseptically added. The medium was mixed and poured in sterile plates (Barrow and Feltham 1993).

2.3.1.6. Starch agar:-

This media consisted of 10 gm of potato starch, 50ml of distilled water and 1000 ml of nutrient agar. The starch was triturated with water to a smooth cream, added to the molted nutrient agar, missed, the medium was sterilized at 115°C for 10 minutes and distributed into sterile Petri-dishes.

2.3.1.7. Urea agar base (oxoid):-

Two point four grams which consisted of 1 gm peptone, 1 gm dextrose, 5 gm NaCl, 1.2gm disodium phosphate, 0.8 gm potassium

dihydrogen phosphate, 0.012 gm phenol red and 15 gm agar were suspended in 95 ml of distilled water and dissolve. The medium was sterilized by autoclaving at 115°C for 20 minutes after adjusting the pH to 6.8 then cooled to 50°C and aseptically introduced 5ml of sterile 40% Urea solution, mixed well, distribute 10ml amounts of media into sterile containers and allowed to set in the slope position.

2.3.1.8. Simmon's citrate agar (oxoid):-

Twenty three gram which consisted of 0.2gm of magnesium sulphate, 0.2gm ammonium dihydrogenphosphate, 0.8 sodium ammonium phosphate, 2gm sodium citrate, 5gm NaCl, 0.8gm bromothymol blue and 15gm agar were suspended in 1 liter of distilled water, boiled to dissolve completely. The medium was sterilized by autoclaving at 121°C for 15 minutes after adjusting the pH to 7.0. Then it was cooled to 50°C and 5ml of sterile 40% Urea solution was aseptically added, mixed well, distributed 10ml amounts into sterile containers and allowed to set in the slope position.

2.3.1.9. Ammonium Salt Sugars (Ass):-

The medium Consisted of 1gm ammonium monohydrogen phosphate, 0.2gm potassium chloride 0.2gm magnesium sulphate, 0.2 gm yeast extract, 20gm agar and 4 ml bromocresol purple.

The solids were added to the water and dissolve by steaming, added the indicator and sterilized at 115°C for 20 minutes, allowed the basal medium to cooled to about 60°C, then the appropriate carbohydrate was added as a sterilized solution to give a final concentration of 1%, mixed and

distributed aseptically into sterile tubes which were inclined so that the medium sets as a slope.

2.3.2. Semi Solid Media:-

2.3.2.1. Motility Medium:-

The fourteen point one grams of medium were suspended in 1 liter of distilled water and brought to boil to dissolve completely; the pH was approximately adjusted to 7.2. The medium was distributed in 3.5ml portions into test tubes containing craigie tubes and the prepared media was sterilized by autoclaving at 115°C for 15 min.

2.3.2.2. Hugh and Liefson's (0.F) medium:-

Two grams of peptone, 5gm NaCl, 0.3gm potassium hydrogen phosphate, 3gm agar were dissolve in 1 liter of distilled water by heating in water bath at 55C° the pH was adjusted to 7.1 and filtered. 15ml of 0.2% aqueous solution of bromothymol blue was added as indicator and sterilized by autoclaving at 115°C for 20 minutes then sterile glucose solution was added aseptically to give final concentration of 1% then 10ml of amounts of medium was aseptically distributed into sterile test tubes after mixing. (Barrow and Feltham 1993).

2.3.3. Liquid Media:-

2.3.3.1. Peptone water:-

Fifteen grams which consisted of 10gm peptone and 5gm NaCl were added to 1 liter of distilled water, mixed well, distributed into final containers after adjusting the pH to 7.4 and sterilized by autoclaving at 121°C for 15 minutes.

2.3.3.2. Peptone water sugar:-

The solution of the specific sugar was prepared by dissolving 10mg of sugar in 90 ml of distilled water and Nine hundreds of peptone water, 10ml of Andrad's indicator adding to the mixture, mixed thoroughly, and distributed into sterile test tubes (containing Durham's tube instead of glucose) then sterilized by autoclaving at 115°C for 10 minutes after adjusting the pH to 7.1-7.3.

2.3.3.3. Methyl red and Vogus Proskauer medium (oxoid):-

The medium Consisted of 5gm peptone, 5gm dispotassium hydrogen phosphate and 1000 ml distilled water which mixed, steamed to dissolve, filter and adjusted the pH to 7.5, and sterilized by autoclaving at 115°C sterile glucose was added, mixed distributed into sterile tubes.

2.3.3.4. Nitrate Broth:-

One gram of the KNO_3 (potassium nitrate) was dissolved in the 1000ml of the nutrient broth and then it was distributed in tubes, and sterilized by autoclaving at 115°C for 20 minutes.

2.3.3.5. Nutrient broth with 10% NaCl:

Nutrient broth with NaCl content increased to 95g for 1000 ml to give the concentration required.

2.4. Reagents:-

All reagents, which were used in this study, were obtained from British Drug House Chemicals, UK (BDH) and prepared according to Barrow and Feltham (1993).

2.4.1. Hydrogen Peroxide:-

This was prepared as 3% aqueous solution, protected from light stored in a cool place, and used for catalase test.

2.4.2. Oxidase test reagent:-

Manufacture by British Drug House, was prepared as fresh solution. To 1% tetramethy-l-P-phenylenediamine aqueous solution, 1% ascorbic acid was added filter paper of 50x50mm was impregnated in the above reagent and dried at 50°C.

2.4.3. Nitrate test reagent:-

Consisted of two reagent (A&B) solution A was consisted of 33% sulpanilic acid which dissolve by heating in 5-N- acetic acid, while solution B was consisted of 0.6% dimethyl-alpha-naphthyl amine, dissolve by gentle heating in 5-N-acetic acid (Barrow& Fleltham, 1993).

2.4.4. Kovac's reagent:-

Consisted of 5gm p-dimethyl amino-benzaaldehyde, 75ml of famyla lcohol, and 25ml concentrated hydrochloric acid. Dissolve the aldehyde in the alcohol by gentle warming in water bath (50-55°C), cooled, added the acid protected from light and stored at 4°C.

2.4.5. V.p test reagent:-

1) Al pha-naphthol solution was consisting of 5% alpha-naphthol in ethanol.

2) KoH 40% solution.

2.4.6. Lugol's Iodine:-

Consisted of 5gm Iodine, and 10gm potassium iodine which dissolve in 100ml distilled water and used after diluted 1/5.

2.4.7. Lead acetate paper:-

Prepared from a filter paper cut into strips of 5-10 mm wide and 50-60 mm long that impregnated with the hot saturated lead acetate solution, dried at 50-60°C and stored in screw- capped containers.

2.5 indicators:-

Was prepared according to (Barrow, feltham 1993)

2.5.1. Andrade's indicator:-

It was used for peptone water sugar medium, by dissolving 5 gram of fuchsine acid in 1000ml distilled water and then 150 ml of alkalin solution of (normal NaOH) was added, mixed and allowed to stand at room temperature with frequent shaking for 24 hours the colour changed from red to brown- additional alkali was added until a straw- yellow colour was attained with the minimum addition of alkali.

2.5.2. Bromthymol blue:-

It was used for citrate medium and (0.F) medium, it was supplied by (BDH) and prepared by dissolving 0.2g of the powder in 100ml distilled water.

2.5.3. Neutral red:-

Product of Hopkir and William Ltd, London.

2. 5.4. Bromocresol purple:-

0.2% in 37ml of 0.05 NaOH added to 63ml water. It was used for (Ass).

2. 5.5. Phenol red:-

It was used for Urea agar base medium; it was supplied by Hopkin and William Ltd. London.

2.6. Methods of sterilization:-

2.6.1. Dry Heat:-

2.6.1.1. Hot air oven:-

This method was used for sterilization of clean glass ware, such as Petri-dishes, pipettes, tubes, flasks, and bottles. The temperature and time of exposure was 160°C for one hour (Stainer *et al.*, 1986).

2.6.1.2. Red Heat:-

It was used for sterilization of wire loops, straight wires and forceps. It was done by holding the object as near as possible to the flame until it became red hot (Cruickshank *et al.*, 1975).

2.6.1.3. Flaming:-

It was used for cotton plugged tubes and glass slides it was done by exposing the object to the direct flame for about half one second.

2.6.2. Moist Heat:-

2.6.2.1. Autoclaving:-

This method was used for sterilizing material or media that will withstand temperatures over 100°C, the temperature-time combinations

used either 155°C (0.69kg/cm²) for 20 minutes or 121°C (1,6kg/cm²) for 15 minutes under pressure of 10 pound, autoclaving at 110°C for 10 minutes was used for sugar media.

2.6.3. Radiation:-

The ultra violet (UV) irradiation was used for sterilizing the media pouring room for 20 minutes.

2.7. Disinfection:-

Phenolic disinfection was used for disinfecting floor, walls, roofs of the Laboratories and 70% alcohol was used for disinfecting the benches.

2.8. Methods for bacterial isolation and identification:-

2.8.1. Aerobic and anaerobic cultivation and purification:-

For isolation of aerobic and anaerobic bacteria, homogenous samples was inoculated on Blood agar and MacConkey agar plates. Plates were incubated at 37°C for 24hrs, for aerobic isolation and 48hrs, for anaerobic isolation. Well isolated colonies of different types were sub-cultured on fresh blood agar and nutrient agar for purification.

2.8.2. General examination:

General morphology – color, shape and size of colonies were examined with the naked eye. Haemolysis on blood agar was observed.

2.8.3. Primary Tests:-

2.8.3.1. Gram staining and microscopy:-

Gram stain was used to study morphology, shape, spores, and gram staining reaction of each isolate. A sterile loop was used to prepare an

emulsion from a single colony in normal saline on a clean slide. A thin smear was made and allowed to dry in air, fixed by flaming and placed on rack. The slide was covered with Lugol's iodine for 30sec., rinsed with water, decolorized by acetone or 70% alcohol and counter stained with diluted carbol fuchsin for 30sec, rinsed water. The slide was dried by blotting with filter paper and examined by bright field microscope under (100x) magnification using oil immersion lens. Gram positive bacteria were blue or purple, gram negative bacteria were red.

2.8.3.2. Catalase test:-

A drop of 3% aqueous solution of hydrogen peroxide was placed on a clean slide. A colony of the test culture, on nutrient agar, was placed on hydrogen peroxide. Gas bubbles which appeared on the surface indicated a positive reaction.

2.8.3.3. Oxidase Test:-

The test was performed by placing the soaked dried filter paper strip on a clean slide. Small amount of fresh test culture smeared on the strip. Positive reaction gave deep purple color on sec.

2.8.3.4. Motility test:-

Inoculated the test culture by straight loop to a depth of 5mm and incubate at 37°C motile bacteria migrated through the medium, which became turbid growth of non motile bacteria was confined to the stab of inoculation.

2.8.3.5. The oxidation fermentation test:-

Two test tubes of Hugh and Leifson's medium were inoculated with test culture and one of them was covered with a layer of sterile paraffin oil to a depth of 1-2cm. The two tubes were incubated at 37°C and examined daily for up to 14 days. The oxidative bacteria gave acid production in the open tube only, while the fermentative bacteria gave acid production in both tubes and alkaline reaction gave blue color.

2.8.3.6. Sugars fermentation test:-

The peptone water sugar was inoculated with test culture incubated at 37°C, and examined daily. Reddish color indicated acid production; if color remained unchanged the organism was considered negative for acid production from sugar media.

2.8.4. Secondary test:-

All these tests were done according to Barrow and Feltham 1993 as follows as:

2.8.4.1. Indole test:-

Peptone water medium was inoculated with test culture and incubated at 37°C for 48hr one ml of Kova's reagent was run down the side of the tube; the positive reaction gave pink ring within a minute.

2.8.4.2. Voges – Proskaver (V.P) test:-

Glucose phosphate medium was inoculated with test culture and incubated at 37°C for 48hr then 0.6ml of 5% alpha. Naphinol solution and 0.2ml of 40% KOH aqueous solution were added to 1ml of cultured media, shaken well, slope and the tubes were examined after 15 minutes in 1hr, a strong red color indicated positive reaction.

2.8.4.3. Hydrogen sulphate production:-

A lead acetate paper was placed into the neck of the tube containing cultured peptone water incubated at 37°C and examined daily for 7 days. Blackening of the paper indicated a positive reaction.

2.8.4.4. Nitrate reduction test:-

Nitrate broth was inoculated with test culture incubated at 37°C for 2 days. Then 1 ml of solution A (33% sulphuric acid) and 1 ml of solution B (0.6% dimethyl- α -naphthylamine in 5-N-acetic acid) were added. Appearance of deep red color indicated a positive reaction. Powdered zinc was added to negative tubes; absence of red color indicated a positive reaction.

2.8.4.5. Urease activity:-

The test bacteria was streaked on urea agar and incubated at 37°C for 7 days. Change in color to pink indicated a positive reaction.

2.8.4.6. Citrate Utilization test:-

The test bacteria was streaked in Simmons' citrate media, and incubated for 7 days at 37°C. An alkaline reaction indicated a positive reaction and the color of the media changed to blue.

2.8.4.7. Digestion of casein:-

Plate of casein agar was inoculated, incubated at 37°C and examined for up to 14 days for clearing of the media around the bacterial growth which indicated a positive reaction.

2.8.4.8. Lecithinase reaction:-

Lecithoritelline agar was inoculated with the test bacteria and incubated at 37°C for 5 days to examine the growth, opalescence and around the growth. The appearance of pearly layer around the colonies constituted a positive reaction.

2.8.4.9. Starch hydrolysis:-

Starch agar was inoculated by test bacteria and incubated at 37c for 5 days then flooded with Lugol's iodine solution, clear colorless zones indicated starch hydrolysis.

2.8.4.10. Ammonium salt sugar test:-

Ammonium salt sugar medium was inoculated with the test bacteria and incubated at 37°C for 7 days, growth and acid production indicated a positive reaction and the color media change from purple to yellow.

2.8.4.11. Growth in media with increased NaCl concentration:

Broth of the required salt concentration was inoculated with the organism to be tested, and incubated at 37°C for 7 days. Salt organisms (halophiles bacteria) showed a good growth in this media, while non salt-tolerant organism showed no growth.

2.9. Method for measurement of Eh (Redox potential) and pH in the samples

2.9.1. Materials:

- 1) Moist snuff
- 2) Dry snuff
- 3) Natron solution

- 4) Sodium nitrate solution
- 5) Cystein hydrochlorid 0.05%

2.9.2: Equipment:

- 1) Eh meter
- 2) pH meter

2.9.3: Preparation of sample:

- (1) 80 gram of moist snuff dissolves in 200ml Distilled water
- (2) 20 gram of dry snuff dissolves in 200ml Distilled water.
- (3) 20 gram of natron powder dissolves in 200ml Distilled water
- (4) 4)20 gram of sodium nitrate dissolve in 200ml Distilled water.

2.9.3. Average time of Eh measurements in the samples:

The Eh measurements were taken before boiling the samples solutions and after boiling about 30 minutes and cooling 15 min. Two minutes were considered as average time for Eh measurements.

CHAPTER THREE

Results

A total number of 66 samples containing 50 sample of moist snuff and 16 samples of dry snuff were collected and subjected to general biochemical analysis as well as bacteriological examination by direct microscopy and culture. Both Gram – positive and Gram – negative bacteria were isolated. All bacteria isolated grow aerobically, and no anaerobic growth was detected. No growth on McConkey agar media. All tested samples showed positive growth for different *Bacillus* species, other isolate of *providencia* species was detected from dry samples only.

3.1 bacteria isolated form Sudanese toombak

Table (1) shows number of Gram positive and Gram negative bacteria isolated.

3.2 types of *Bacillus* species is;lated from toombak

Table (2) shows the number of types of the *Bacillus* species isolated form toombak.

3.2.1. *Bacillus* specie:-

3.2.1.1 Cultural characteristics of isolate *Bacillus* species:-

Microscopic examination of isolates revealed Gram – positive short and /or long rods, occurring singly or in diplo – bacillary form, endospores were terminal, sub terminal or central. More than one species of *Bacillus* was isolated from one sample, twelve different species were isolated in this study; all *Bacillus* species were motile except some isolates of *B. mycoides*

and *B. subtilis*. A heavy turbidity was produced by all the isolates in both media following over – night incubation in broth cultures, a pellicle was formed on the surface and was easily dispersible by shaking.

On nutrient agar colonies were very large, rough and flat with curled edges, raised, dull grayish – white. Opaque colonies were also observed, colonies were small, round and flat with white color.

On blood agar colonies of *Bacillus* produced complete haemolysis. The two strains of *Bacillus mycoides* produced two different types of colonies in blood and nutrient agar plates. The colonies of one strain were round, large raised, rhizoid and spreading in granular appearance in grayish to white color, while the colonies of other strain seem to be round small in white color. Both strains produce mucoid slimy colonies on agar.

3.2.1.2 Biochemical Properties of Bacillus Species Isolate:-

Table (3) shows the biochemical properties of *Bacillus* species isolated from dry and moist snuff in Sudan.

All *Bacillus* species formed acid from fermentation of glucose except *B. brevis* and *B. sphaericus*, The ability to ferment other sugars varied from species to other. Lecithinase reaction was positive only in *B. cereus*, *B. thuringiensis* and *B. mycoides*. All *Bacillus* species were positive for starch hydrolysis except *B. brevis*, *B. sphaericus*, *B. pantothenicus* and some isolates of *B. mycoides*. Utilization of citrate was positive in *B. cereus*, *B. thuringiensis* and *B. subtilis*, *B. mycoides*, *B. megaterium*, *B. coagulans* and *B. licheniformis*. Indole test was positive only in *B. alvei*. Nitrate reduction

was positive in all species except some isolates of *B. mycoides*, *B. sphaericus* and *B. megaterium*.

3.3. providencia. rettgeri:-

3.3.1. Cultural characteristics of providencia. rettgeri:-

The colony on nutrient agar was flat, slightly raised, swarming surface with grayish color.

3.3.2. Biochemical properties of providencia rettgeri:-

Table 4 shows the biochemical properties of *providencia rettgeri* isolated from dry snuff. The organism is motile, fermented the carbohydrates and does not utilize the citrate, the nitrate and the indole reaction is positive

3.4 The measurements of redox potential (Eh) in the samples

3.4.1 moist toombak

3.4.1.1 Table (5) shows the measurements of Eh in moist toombak solution.

3.4.1.2 Table (9) shows the measurements of Eh in moist toombak solution after boiling about 30 min and cooling in 15 min

3.4.1.3 Table (11) shows the measurements of Eh in moist toombak solution after addition of the 3ml cysteine hydrochloride.

3.4.2 Dry toombak

3.4.2.1 Table (6) shows the measurements of Eh in dry toombak solution.

3.4.2.2 Table (10) shows the measurements of Eh in a mixture of dry toombak and natron solution after boiling and cooling about 15 min.

3.4.3 Natron solution

3.4.3.1 Table (7) shows the measurements of Eh in natron solution.

3.4.4 Sodium Nitrate solution

3.4.4.1 Table (8) shows the measurements of Eh in Sodium nitrate solution

Table (1) shows the number of isolates and total of Gram positive and Gram-negative isolate.

Tapes of bacteria isolated	Number of each isolate	Hundred percentage of each isolate
Gram positive	193	%98.9
Gram negative	2	1.5%
Total	195	100%

Table (2) shows the total number and the hundred percentage of each isolate:-

Types of Bacillus species	Number of isolate	Hundred percentage
<i>Bacillus.amyloliquefaciens</i>	39	20.2%
<i>B.mycoid (motility +ve)</i>	21	10.8%
<i>B.subtilis (motility +ve)</i>	30	15.5%
<i>B.subtilis (motility -ve)</i>	7	3.6%
<i>Bacillus .cereus</i>	12	6.2%
<i>Bacillus. alvei</i>	2	1.0%
<i>Bacillus .thuringiensis</i>	17	8.8%
<i>Bacillus .mycoid (motility-ve)</i>	9	4.7%
<i>Bacillus. brevis</i>	7	3.6%
<i>Bacillus. sphaericus</i>	16	8.2%
<i>Bacillus.megaterium</i>	14	7.3%
<i>Bacillus.coagulans</i>	9	4.7%
<i>Bacillus. licheniformis</i>	9	4.7%
<i>Bacillus.pantothenicus</i>	1	0.5%
Total	193	100.0%

Table (3) shows the biochemical properties of Bacillus species

	oxidase	vp	Lecith*	Motility	starch	glucose	Mannose	Galactose	xylose	raffinose	Salicin	casin	nitrate	urease	citrate	10%l
	+	+	+	+	+	+	-	-	-	-	+	+	+	±	±	±
	+	+	+	+	+	+	±	-	-	-	+	+	+	-	+	+
	+	+	+	±	±	+	-	±	-	-	+	+	±	±	±	±
	+	+	-	±	+	+	+	+	+	+	+	+	+	-	+	+
	-	-	-	+	+	+	+	+	+	±	+	+	±	±	+	-
	-	+	-	+	+	+	+	-	+	+	+	+	+	-	+	-
	+	-	-	+	-	-	-	-	-	-	-	+	±	±	±	-
	+	+	-	+	+	+	+	-	-	+	-	+	-	+	-	-
ns	-	+	-	+	+	+	+	±	+	+	+	+	+	-	+	+
	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
			-	+	-	+	+	-	-	-	+	+	+	-	-	+

* Lecithovitellin reaction

Table (4) shows the Biochemical reactions *Providencia rettgi*

Biochemical reactions	<i>Providencia rettgi</i>
Glucose	+
Mannose	+
Galactose	+
Salicin	+
Xylose	-
Raffinose	-
Mannitol	+
Sucrose	+
Sorbitol	-
Urease	+
Citrate	-
10%NaCL	-
Starch	-
Indole	+
Motility	+

Table (5) shows the measurements of Eh in moist snuff solution:-

Time(min)	Eh in moist snuff(m/v)
1,2	- 150
4,1	- 157
6,1	- 171
1,8	- 183
1,10	-195
1,12	- 195,7
1,14	-201
1,16	-205
1,18	-208
1,20	-212,5
1,22	-214,3
1,24	216,9
1,26	-219,4
1,28	-221
1,30	-223,7
1,32	-225,6

1,34	-227,5
1,36	-229,3
1,38	-230,9
1,40	-232,5
1,42	-233,8
1,44	-235,4
1,46	-236,6
1,48	-238
1,50	-239,2
1,52	-245,5
1,54	-241,5
1,56	-242,6
1,58	-243,7
2,00	-244,9
2,2	-245,8
2,4	-246,7
2,6	-247,7
2,8	-248,6

2,10	-249,4
2,12	-250,2
2,14	-251,1
2,16	-251,8
2,18	-252,6
2,20	-253,3
2,22	-254
2,24	-254,6
2,26	-255,3
2,28	-255,6
2,30	-256,6
2,32	-257,1
2,34	-257,7
2,36	-258,3
2,38	-258,9
2,40	-259,4
2,42	-259,9
2,44	-260,4

2,46	-260,9
2,48	-261,4
2,50	-261,9
2,52	-262,3
2,54	-262,8
2,56	-263,2
2,58	-263,6
3,00	-264
3,2	-264,4
3,4	-264,8
3,6	-265,2
3,8	-265,6
3,10	-266
3,12	-266,3
1,14	-266,6
3,16	-267
3,18	-267,4

Table (6) shows the measurements of Eh in dry snuff solution:-

Time (min)	Eh in dry snuff (m/v)
3,24	-90
3,26	-100
3,28	-104,8
3,30	-108,9
3,32	-111,8
3,34	-114,4
3,36	-116,2
3,38	-117,6
3,40	-11,8
3,42	-120,1
3,44	-121
3,46	-121,8
3,48	-122,7
3,50	-123,4
3,52	-124,2

3,54	-124,9
3,56	-125,5
3,58	-126,2
4,00	-126,6
4,2	-127,1
4,4	-127,8
4,6	-128,2
4,8	-128,6
4,10	-129
4,12	-129,1
4,14	-129,5
4,16	-129,9
4,18	-130,4
4,20	-130,8
4,22	-131,3
4,24	-131,5

4,26	-132
4,28	-132,4
4,30	-132,7
4,32	-133
4,34	-133,4
4,36	-133,7
4,38	134,1-
4,40	134,4-

Table (7) shows the measurements of Eh in Natron solution:-

Time (min)	Eh in Natron (m/v)
4,36	+42,4
4,38	+46,00
4,40	+49,5
4,42	+52,1
4,44	+54,1
4,46	+55,9
4,48	+57,3
4,50	+58,6
4,52	+59,9
4,54	+61,00
4,56	+61,9
4,58	+62,8
5,00	+63,7
5,2	+64,4
5,4	+65,2

5,6	+65,9
5,8	+66,6
5,10	+67,2
5,12	+67,8
5,14	+68,3
5,16	+68,8
5,18	+69,3
5,20	+69,9
5,22	+70,4
5,24	+70,9
5,26	+71,3
5,28	+71,8
5,30	+72,2
5,32	+72,7
5,34	+73,00
5,36	+73,4

5,38	+73,8
5,40	+74,2
5,42	+74,4
5,44	+74,6
5,46	+74,8
5,48	+75,00
5,50	+75,2
5,52	+75,5
5,54	+75,8
5,56	+76,1
5,58	+76,4
6,00	+76,6

Table (8) shows the measurements of Eh in Sodium Nitrate Solution:-

Time (min)	Eh in sodium nitrate (m/v)
6 ,19	+303,3
6 ,21	+305,1
6 ,23	+308,9
6,25	+310,5
6,27	=316,9

Table (9) shows the measurements of Eh in moist Snuff Solution after Boiling about 30 min and cooling 10 min

Time (min)	Eh in moist snuff after boiling (m/v)
2,10	-304,5
2,12	-307,5
2,14	-308,3
2,16	-308,8
2,18	-309,1
2,20	-309,5
2,22	-309,7
2,24	-310,00
2,26	-310,3
2,28	-310,6
2,30	-310,8
2,32	-311,1
2,34	-311,3
2,36	-311,6
2,38	-311,9

Table (10) shows the measurements Of Eh in mix of Dry Snuff and Natron Solution after boiling and cooling 15min:-

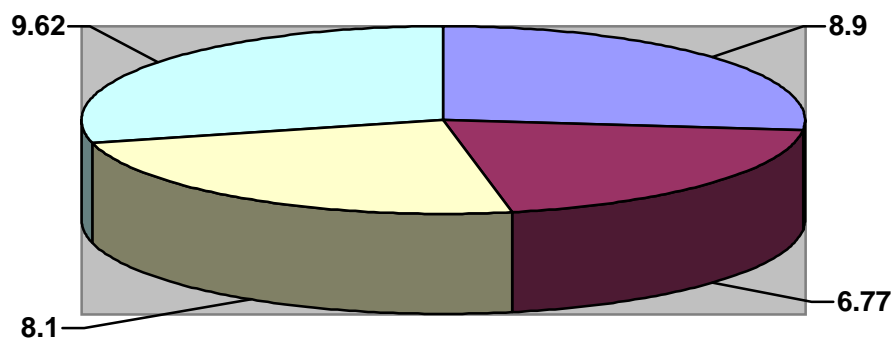
Time (min)	Eh in mixture of dry snuff, natron(m/v)
4,23	-290,00
4,25	-294,3
4,27	-295,7
4,29	-296,6
4,31	-297,1
4,33	-297,4
4,35	-297,5
4,37	-297,6
4,39	-297,9
4,41	-298,1

Table (11) shows the measurements of Eh in moist snuff Solution after addition of 3ml cystein hydrochloride:-

Time (min)	Eh in moist snuff with cystein Hcl(m/v)
2,40	-312,2
2,42	-312,4
2,44	-312,8
2,46	-313,1
2,48	-313,3
2,50	-313,5
2,52	-313,8
2,54	-314,3
2,56	-314,7
2,58	-315,2
3,00	-315,8
3,2	-316,3
3,4	-317,7
3,6	-318,2
3,8	-319,7

The addition of cystein Hcl to moist snuff solution the be come red indicate that the media become more reducible.

fig .1. Meantments of pH in the samples



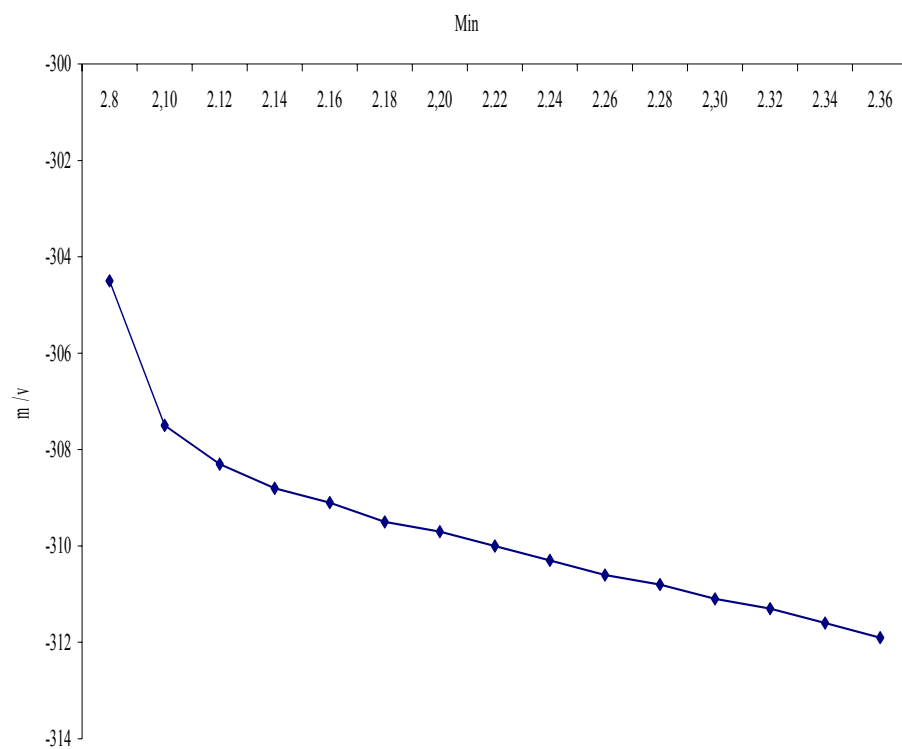
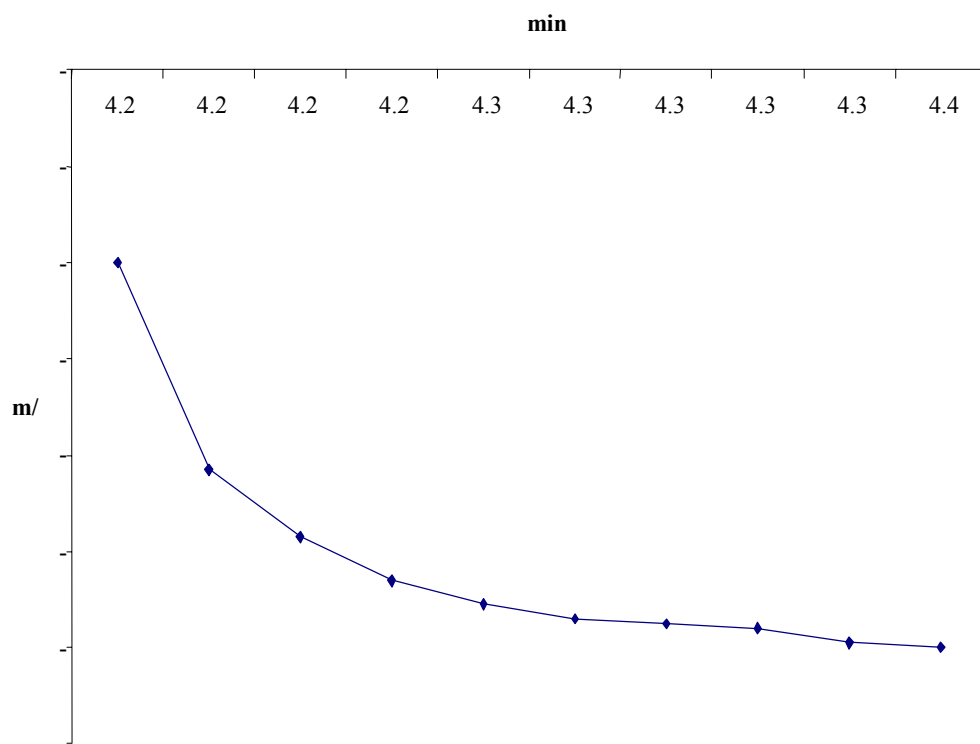


Fig. 2. The Eh of moist snuff after addition of 3ml cystein hydrochlorid 0.05%



Fi3. The Eh of mixture dry snuff and ntrone after boilling

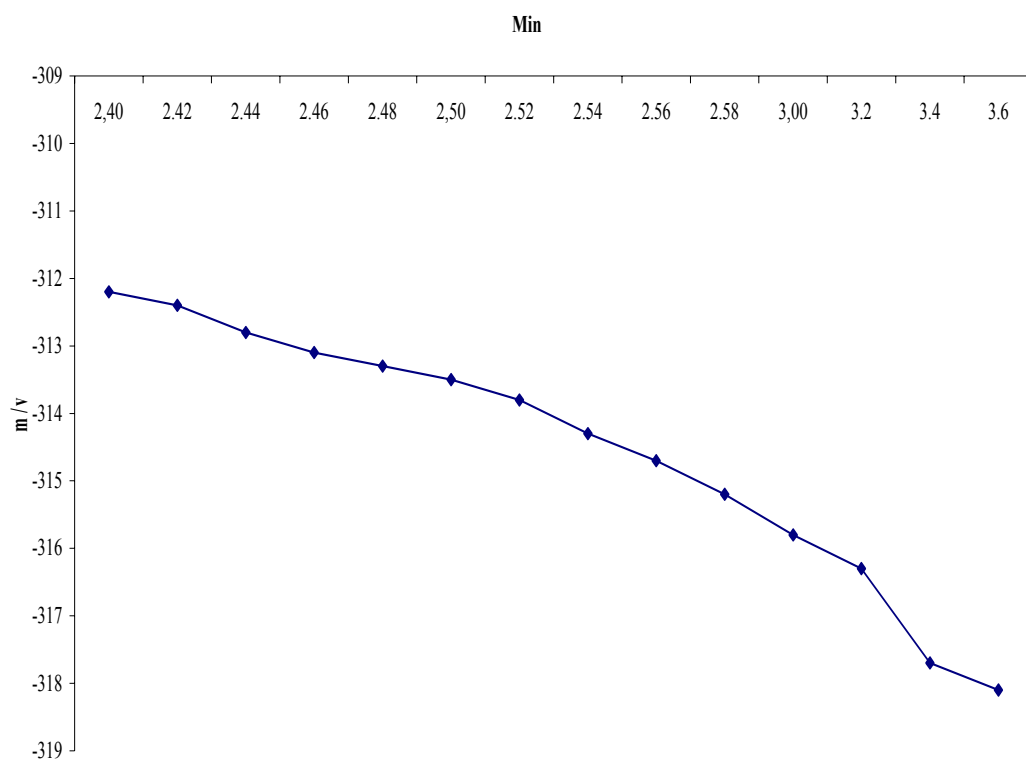


Fig. 4. The Eh of moist snuff after boiling about 30 min and cooling about 15 min

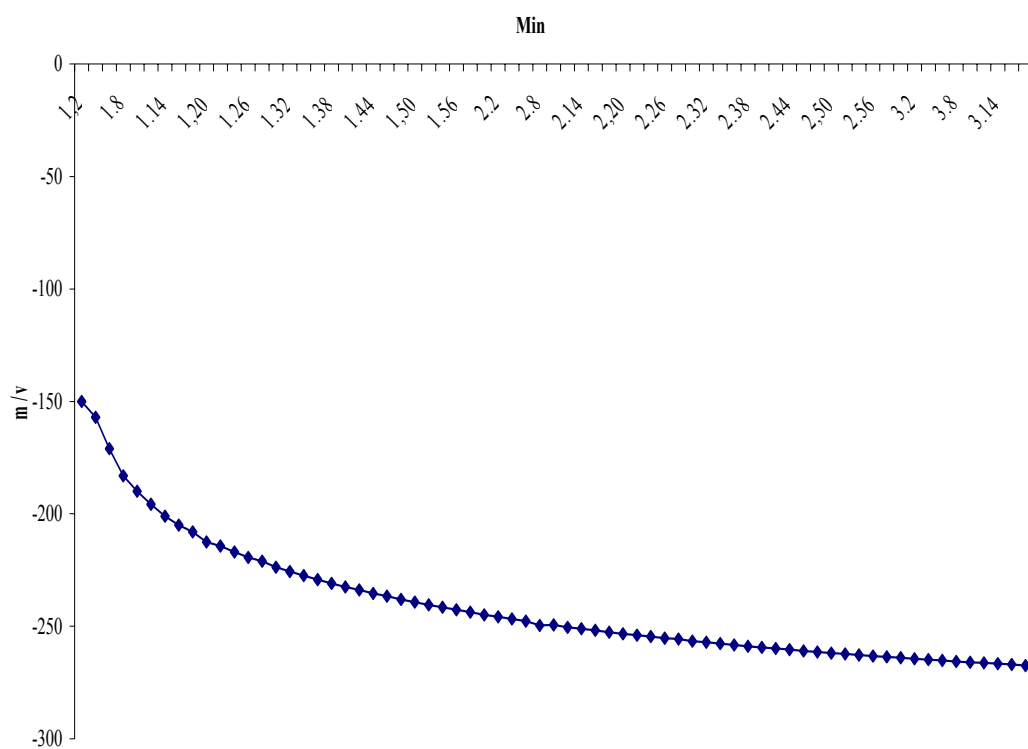


Fig. 5. Measurement of Eh in the moist snuff

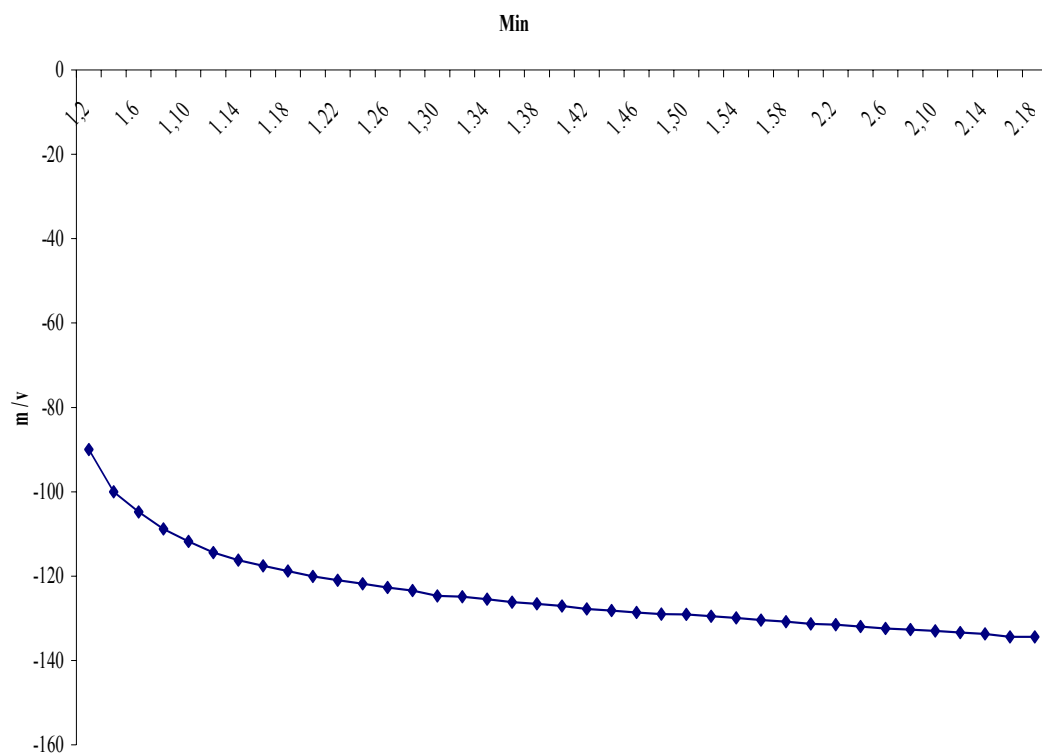


Fig. 6. Measurement of Eh in the dry snuff

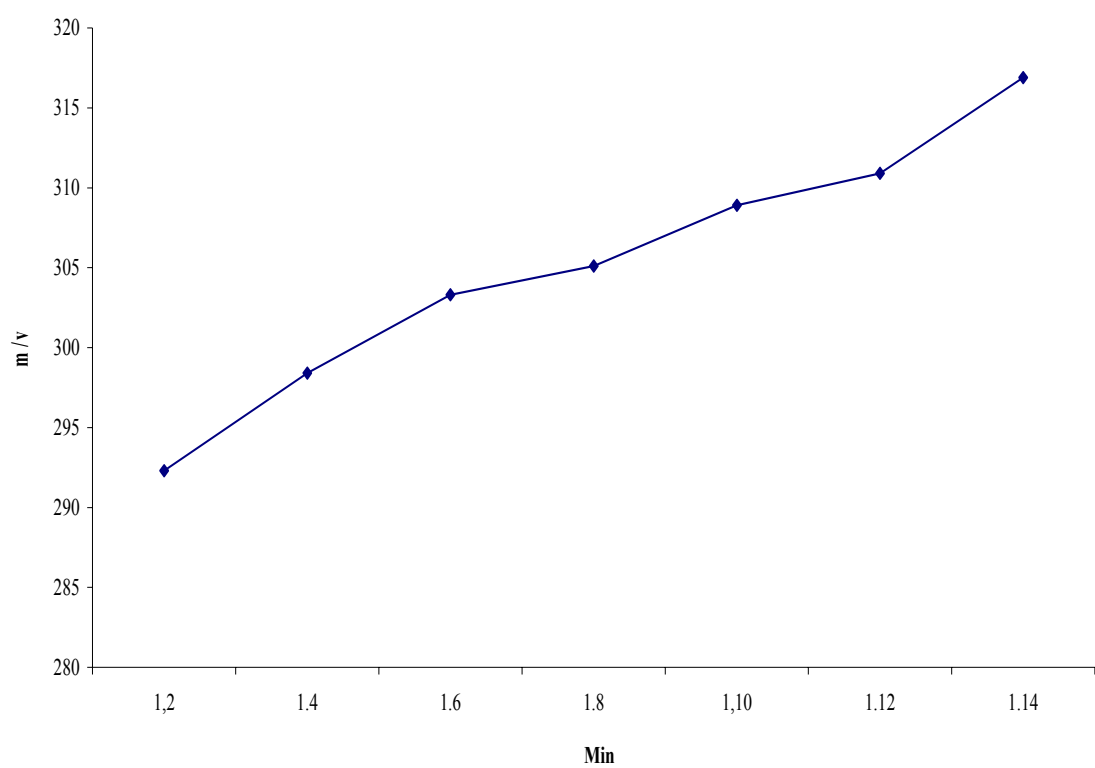
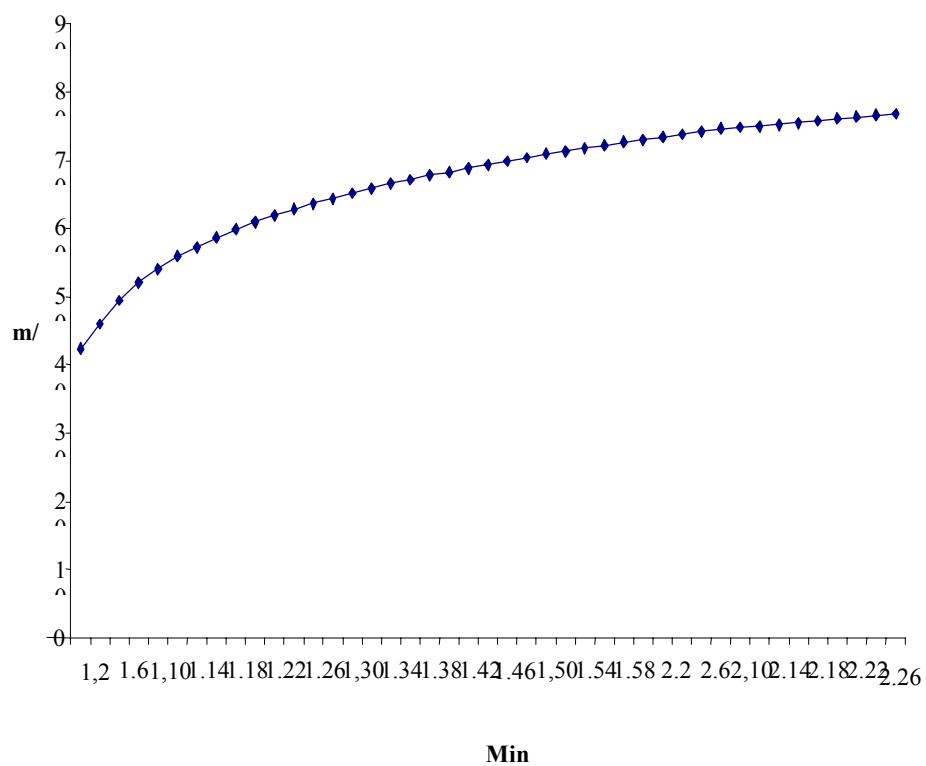


Fig. 7. Measurement of Eh in the sodium nitrate



Fi8 Measurement of Eh in the natron



Fig. 9: B. cereus on nutrient agar



Fig. 10: B. cereus on blood agar



Fig. 11: B. thuringiensis on nutrient agar

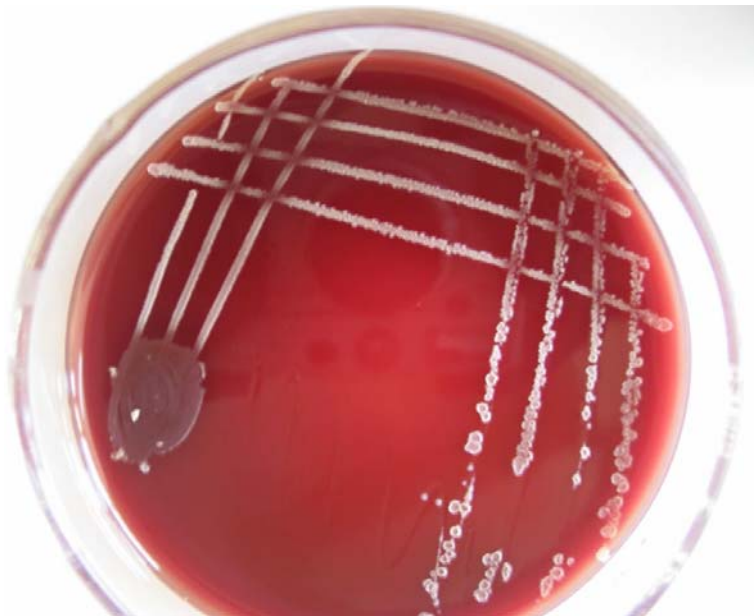


Fig. 12: B. thuringiensis on blood agar



Fig. 13: B. mycoides on nutrient agar



Fig. 14: B. mycoides on blood agar



Fig. 15: B. subtilis on nutrient agar

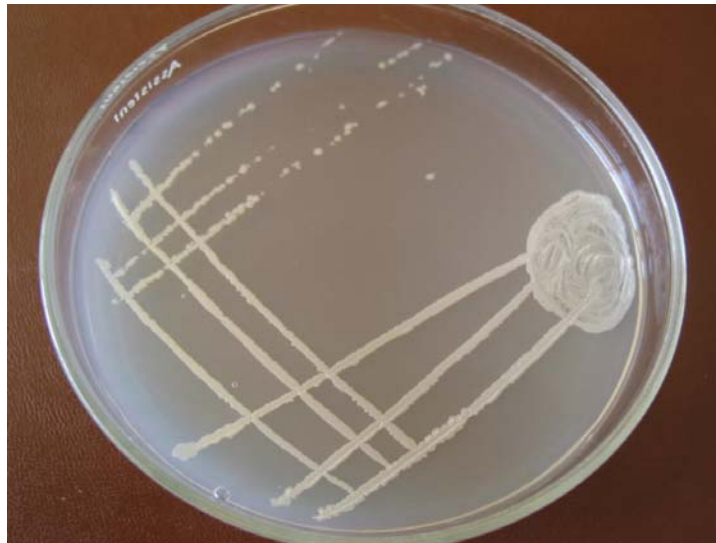


Fig. 16: B. sphaericus on nutrient agar

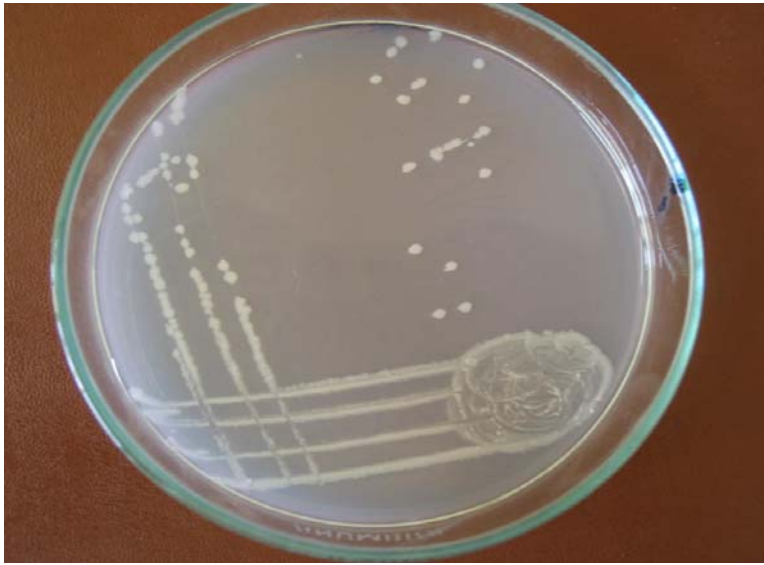


Fig. 17: B. alvei on nutrient agar

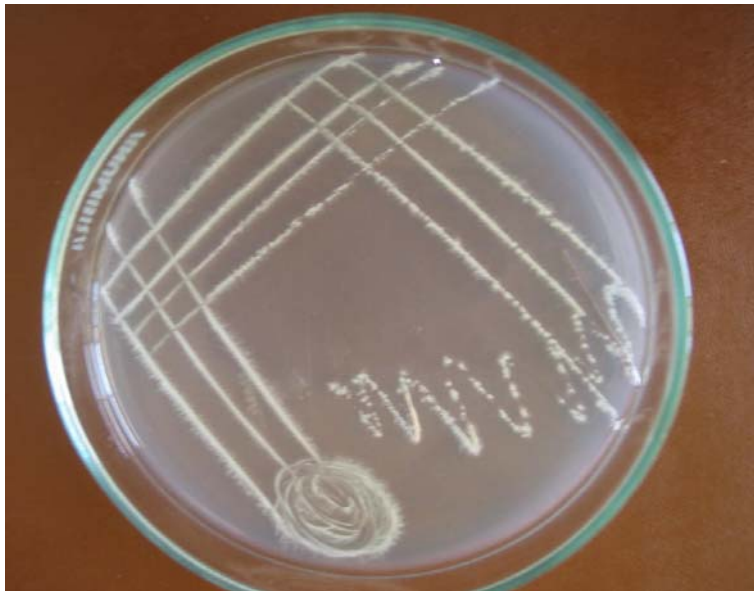


Fig. 18: B. amylichformis on nutrient agar



Fig. 19. Diagram of moist snuff



Fig. 20. Diagram of dry snuff



Fig. 21. Diagram of addition of natron to dry snuff

CHAPTER FOUR

Discussion

Toombak is a type of snuff used extensively in the North of Sudan by virtually non-smoking, non-drinking population. The use of snuff is increased in last years in the world so to decrease the rates of the lung cancer, which caused by smoking (Kuulasmaa *et al.*, 2000).

The widespread use of smokeless tobacco has been prompted concerning to the development of oral lesions in long-term users. The association between smokeless tobacco use and the development of oral lesions is well established (Greath *et al.*, 1991). It is well known that snuff causes changes of the oral mucosa, and that the clinical appearance may vary with differences in consumption factors (Holmstrup *et al.*, 1988 and Poulson *et al.*, 1984). The placement of the quid had a relatively significant impact up on the clinical appearance of the mucosal changes. Number of years with regular snuff habit seemed to have less impact than daily exposure to snuff in terms of hour using snuff daily, and grams of snuff used daily (Roed *et al.*, 1973).

The incidence rates of oral cancer may vary in different parts of the world. The highest rates have been reported as occurring in south Asian countries, where oral cancers constitute 4 to 45% of all cancers. In contrast in North America and Europe 3 to 4% of all cancers are oral neoplasms (Boring *et al.*, 1993). In Asia, the high incidence of oral cancers linked to the habit of chewing tobacco or betel nut with tobacco (Pindborg, 1980; IARC, 1985 and USDHHS, 1986). While in the north of America and

Western Europe, the oral cancer is primarily associated with smoking and /or alcohol consumption (IARC, 1986 and US surgeon general, 1989). Reports from South Africa have linked oral cancer with the use of snuff (Pindborg, 1980). The high prevalence of cancer of the lower lip and of the buccal cavity in high proportion of cancers in men, the greater relative frequency of carcinomas among the tribes in the north of Sudan. All previous studies indicating that toombak is a relatively higher proportion of oral cancer among women, among whom toombak is not known to be widely used .Consumption of toombak among women is unreliable because of religious and cultural influences, furthermore the degree of exposure is known to be different in women and in men .Women are known to start the use of toombak at a much older age and hold the saffa in the buccal sulcus and used smaller quids with change the site of quid more frequently(Wynder *et al.*,1957).

Early reports showed a possible association between the toombak and oral and esophageal cancer were based on clinical and descriptive epidemiological studies (Idris *et al.*, 1995).

Only one, descriptive study from the Sudan examined the risk of cancer in various intra-oral sites in relation to toombak use, in some detail (EL-Besheir *et al.*, 1989), these workers reported a close association between the tumor site and the area in which the saffa was habitually placed, that is the floor the mouth, lower gingivolabial sulcus, and gingivo buccal sulcus. The risk of cancer affecting the floor of mouth was even higher, toombak being frequently retained in this site. So far two descriptive studies have linked the use of toombak with the etiology of esophageal

cancer (Babekir *et al.*, 1989). The strongest and most abundant carcinogens that occur in smokeless tobacco are the tobacco-specific nitrosamine (TSNA), namely, N-nitrosornicotine (NNN) and 4-(methyl-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK) and its reduction product 4-(methyl nitrosamine)-1-(3-pyridyl)-1-butanol (NNAL) (Hecht and Hoffmann, 1988).

Tumorigenicity studies demonstrated that application of a mixture of NNN and NNK to the rat oral cavity induces tumor in these tissue (Hecht *et al.*, 1986). We have estimated the levels of TSNA in toombak and the saliva of toombak users (Idris *et al.* 1991; Prokopczyk *et al.*, 1994) toombak and saliva of toombak users were found to contain high levels of NNN and NNK. These levels were significantly higher than those observed in North American or West European snuff (Murphy *et al.* 1994).

The unusual high level of TSNA in toombak and saliva of toombak users and TSNA metabolites in the urine of toombak users, combined with both previous and present epidemiological findings strongly support an etiological association between toombak dipping and oral cancer.

TSNA and N-nitrosamine acids are the major classes of carcinogens in smokeless tobacco and betel quid tobacco mixtures, most are formed after the harvesting of tobacco, during fermentation and ageing. The major precursor of TSNA is nitrate, which is reduced to nitrite, the nitrosating agent for the tobacco alkaloid. The higher alkaloid concentration in the leave, the higher the concentration of TSNA in smokeless tobacco (Djordjevic *et al.*, 1989).

Sudanese toombak contains high concentrations of (TSNA), especially 4-(methyl-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), the most

potent carcinogenic nitrosamine among (TSNA) which contains 0.15mg/g while the level of N-nitrosornicotine (NNN) 0.014mg/g (Brunnemann *et al.*, 1986). The high level of TSNA in Sudanese snuff could partly be due to the use of *N.rustica* in its preparation, NNN and NNK levels in *N.rustica* have been reported to be much higher than *N.tobaccum* (Bhide *et al.*, 1987). Other factors may be the treatment with natron and subsequent storage before use.

Thus a reduction of nicotine and of the minor alkaloid will effectively lower the TSNA levels in the consumer product. Alkaloid levels in tobacco can be reduced by supercritical extraction with carbon dioxide (Grubbs *et al.*, 1987) or by bacterial degradation of nitrate that leads to reduced forms of nitrogen (Djordjevic *et al.*, 1989). The presence of bacteria in smokeless tobacco may increase the level of the nitrogen by repeat habitual. Although there is a lack of experimental data on reduction of N-nitrosamine acids which can assume that a reduction of nitrate content of the tobacco will also inhibit the formation of these compounds in smokeless tobacco.

The major types of the isolated bacteria from toombak in this study were *Bacillus* species. The members of this genus were known to be more stable at environmental pH up to 11. It can form endospores out of the exponential phase of growth due to nutrient depletion (Kenneth, 2005).

The alkaline pH in toombak due to the addition of natron, prevent the growth of cocci and cocci bacilli which we couldn't isolate during this study. Gram negative bacteria were couldn't isolate throughout this study due to lack of some substances and suitable pH.

Total of four *Bacillus* species i.e *B.megaterium*, *B brevis*, *B licheniformis* and *B.subtilis* were isolated from snuff in this study and from commercially chewing tobacco in United States (Iseael and Gerald, 2002). In addition, the latter authors were able to isolate *B.pumilus* from chewing tobacco .Such bacteria could isolate from snuff during this study. In contrast eight *Bacillus* species were isolates from Sudanese snuff only. These variations in isolates could be attributed to the differences in manufacturing and processing between the toombak in the Sudan and chewing tobacco in United States.

Most of *Bacillus* species isolated in this study were able to reduce nitrate to nitrite, thus providing more amount of nitrogen. With repeated habitual toombak using, this would lead to increase the level of nitrosamine that found in the saliva of toombak users (Idris *et al.*, 1991).

The redox potential (Eh) is a significant factor for the growth anaerobic bacteria. These bacteria grow readily in special media and conditions containing reduction factor that leads to high negative Eh. This situation was found available in toombak due to the presence of the reducing agent, nitrosamine, and the agent that does not exist in the base of the plant .Moreover, factors like boiling and cooling for 30 and 15 respectively and adding of cystein hydrochloride to moist toombak increase the reduction level of Eh.

The level of pH in toombak creates a suitable media to germinate the bacillus spores when placed on the oral mucosa. This leads to the release of virulence factors such as proteinases. These activate oral kartinocytes and kinin pathway causing plasma exudation and tissue injury. This situation

together with the high Eh of toombak and predisposing factors like ingerint create anaerobic condition leading to growth of anaerobic bacteria. This finding may explain the failure to isolate anaerobic bacteria from toombak samples due to absence of the other stated factors.

In conclusion, rapid delivery of nicotine from snuff products with long term use of snuff can lead to a number of adverse health effects including oral cancer, cardiovascular diseases and gingival disease. It is important that, the public health community considers oral snuff use as a burden on public health in the same way as cigarette smoking.

Natron plays an important in nicotine absorption to blood stream by increasing the level of pH .The processing of toombak is not hygienic leads to bacterial contamination .The fermentation process of toombak may increase the growth of many types of bacteria, but the drying of the leaves, and addition of natron may kill the vegetative cells.

Recommendations:

- (1) Stop using toombak due to bad impact on the health, social, and ecomical situations.
- (2) Many investigations about effects of toombak on the buccal cavity, dental hygiene and related diseases can be done.
- (3) More microbiological, chemical, pharmalogical studies on toombak are needed.
- (4) Use the toombak in anther way, for example extraction of alcohol and others alkaloids which may be used in manufacturing.

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